REVERSIBLE DISSOCIATION OF PIG LIVER GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE BY ADENOSINE TRIPHOSPHATE

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ABSTRACT

REVERSIBLE DISSOCIATION OF PIG LIVER GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE BY ADENOSINE TRIPHOSPHATE

By

Kathryn DePree Mascaro

Glyceraldehyde 3-P dehydrogenase is inactivated and dissociated by ATP at 0°. The effects of incubation time, EDTA, protein concentration, pH, ATP concentration, mercaptoethanol concentration and temperature on this inactivation and dissociation were measured by the oxidative phosphorylation assay and by sucrose density gradient centrifugation respectively. Optimal conditions for inactivation and dissociation were shown to be 12 hours incubation, 0.1 mg/ml protein, pH 7.5 to 9.0, 15 mM ATP and 0°. The tetrameric enzyme dissociated to a dimer with a sedimentation coefficient of 4.9 to 5.2 S. If produced in the presence of 0.1 M EDTA and 40 to 80 mM mercaptoethanol, the dimer was active (50 per cent as much activity per mg protein as the tetramer). At low glyceraldehyde 3-P dehydrogenase concentrations (0.025 mg/ml), the major dissociation product was inactive monomers.

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By warming to 25° for only five minutes, glyceraldehyde 3-P dehydrogenase dimers could be reactivated as measured by the oxidative phosphorylation assay. Reactivation required 0.1 M EDTA and 80 mM mercaptoethanol. Adding NAD increased inactivation; adding sucrose decreased it. In the absence of ATP, the reactivated enzyme was a reassociated tetramer with a sedimentation coefficient of 7.8 S as measured by sucrose density gradient centrifugation. This sedimentation coefficient is the same as that of native glyceraldehyde 3-P dehydrogenase.

The amino acid composition of the enzyme was obtained by analysis of the acid-hydrolysate in a commercial amino acid analyzer. The sulfhydryl reactivity of native glyceraldehyde 3-P dehydrogenase was studied by the titration of sulfhydryl groups with 5, 5'-dithiobis-(2-nitrobenzoic acid). Both the amino acid composition and the sulfhydryl reactivity were very similar to those of other glyceraldehyde 3-P dehydrogenases that have been studied.

The requirement for EDTA in the production of active dimers and in reactivation suggested that glyceraldehyde 3-P dehydrogenase is sensitive to metal ions and that a metal ion(s) may be present on the crystalline enzyme. The possibility that this metal ion is zinc and the effects such a metal ion could have on glyceraldehyde 3-P dehydrogenase were discussed at length.

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The purification procedure for pig liver glyceraldehyde 3-P dehydrogenase developed by Dr. Dagher of this laboratory was confirmed and expanded to a larger scale.

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LIST OF ABBREVIATIONS

BSA -- bovine serum albumin

cAMP -- adenosine 3',5'-cyclic monophosphate

Cys -- cysteine

DPGA -- 1,3-diphosphoglycerate

DTNB -- 5,5'-dithiobis-(2-nitrobenzoic acid)

E -- enzyme

GAPD -- glyceraldehyde 3-phosphate dehydrogenase

G3P -- glyceraldehyde 3-phosphate

His -- histidine

Lys -- lysine

ORD -- optical rotary dispersion

PCMB -- p-chloromercuribenzoate

PGA -- phosphoglyceric acid

P_i -- inorganic phosphate

SDS -- sodium dodecyl sulfate

INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase phosphorylating, EC 1.2.1.12) is an enzyme in glycolysis. It catalyzes the only oxidative step in the pathway and produces one of the two phosphorylated intermediates of glycolysis that can be used directly to make ATP. The enzyme has been isolated from bacteria, fishes, birds, mammals (Allison and Kaplan, 1964) and plants (Duggleby and Dennis, 1974). From all sources tested, except plants (where three different GAPDs are found [Rosenberg and Arnon, 1955; Fuller and Gibbs, 1959; Hageman and Arnon, 1955]), GAPD is a tetramer of approximately 150,000 daltons which binds one NAD per monomer (Watson, Duee and Mercer, 1972). Because of its relative ease of preparation in large quantities, its importance as a model for oxidative phosphorylation, its highly reactive sulfhydryl groups, the cooperativity of its enzyme-coenzyme interactions, its multiple activities and its subunit-subunit interactions, GAPD has been a favorite enzyme for chemical, physical and kinetic investigations.

Since few studies had been reported on GAPD from a gluconeogenic tissue, the aim of this research was to examine pig liver GAPD in hopes of gaining some understanding of how liver (gluconeogenic) GAPD differs from muscle (glycolytic) GAPD. The examination consisted of studies on the dissociation-association properties and on the amino acid composition.

Reversible dissociation and inactivation of GAPD by ATP at 0° had been demonstrated for the rabbit muscle (Constantinides and Deal, 1969) and yeast enzymes (Stancel and Deal, 1969). Dr. Dagher of this laboratory attempted to demonstrate similar behavior by pig liver GAPD, but was only partially successful; dissociation and inactivation were difficult to achieve reproducibly and little reactivation was observed. Since $ZnCl_2$ precipitation was a step in the purification procedure for pig liver GAPD (Dagher and Deal, in preparation) and Keleti found zinc bound to several GAPDs (Keleti et al., 1962), we reasoned that zinc might be bound to the enzyme, causing it to exhibit unexpected stability to inactivation, dissociation and reactivation. Consequently, we added 0.1 M EDTA to the reaction mixture in some preliminary studies on the dissociation of pig liver GAPD by dilution. The results of these experiments indicated that GAPD dissociated more readily in the presence of EDTA than in its absence. We then decided to re-attempt the ATP dissociation using

EDTA. These experiments led to a characterization of the reversible dissociation and inactivation by ATP at 0° in the presence of EDTA.

Amino acid compositions often reflect the differences between various enzymes because the primary sequence determines the enzyme's structure and consequently its function. Thus a difference in amino acid composition might be expected for muscle and liver GAPDs which perform different functions *in vivo*; muscle GAPD mostly catalyzes oxidative phosphorylation because muscle is a glycolytic tissue, while liver GAPD catalyzes both oxidative phosphorylation and reductive dephosphorylation because liver is a gluconeogenic tissue. In hopes of finding such a difference, the amino acid composition of pig liver GAPD and the reactivity of the sulfhydryl groups in the native enzyme were studied, despite the fact that all mammalian GAPDs studied in detail thus far have nearly identical amino acid sequences (Harris, 1970) and that no major structural differences have yet been found between muscle and liver GAPDs (Bondi, Watkins and Kirtley, 1969; Smith and Velick, 1972; Lambert and Perham, 1974).

LITERATURE REVIEW

REACTIONS CATALYZED BY GLYCERALDEHYDE 3-P DEHYDROGENASE. Mechanism for Oxidative Phosphorylation and Reductive Dephosphorylation. The main physiological role of GAPD is to catalyze oxidative phosphorylation and reductive dephosphorylation. The general mechanism proposed for these two reactions by Duggleby and Dennis (1974) is shown in Figure 1.

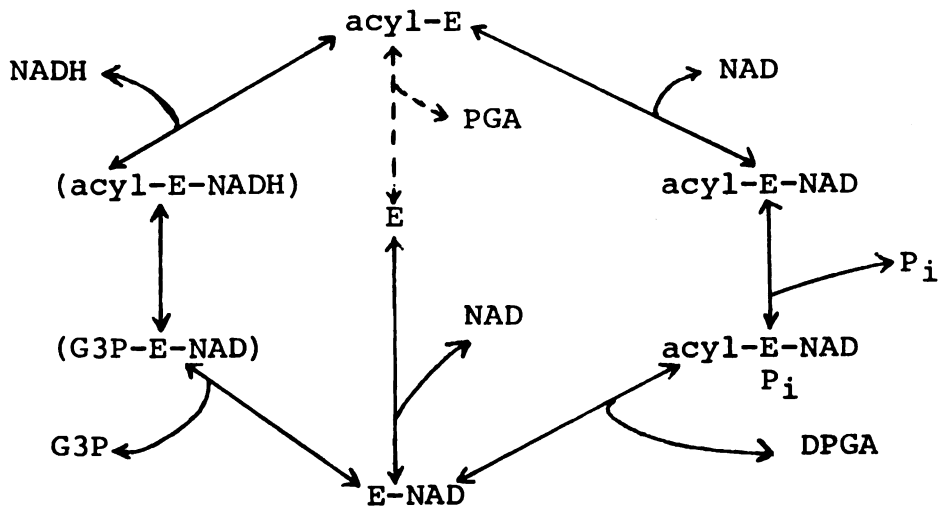

Figure 1. General Mechanism for Oxidative Phosphorylation and Reductive Dephosphorylation

This mechanism is based on the results of studies of pea seedling GAPD (Duggleby and Dennis, 1974), lobster and sturgeon muscle GAPDs (Trentham, 1971) and rabbit muscle and liver GAPDs (Smith and Velick, 1972).

Oxidative phosphorylation involves the formation of an acyl enzyme thioester produced by NAD oxidation of a presumed thiohemiacetal adduct of G3P and GAPD (Racker, 1965); hydrogen is transferred directly from the 1-position of G3P to NAD (Allison, Connors and Parker, 1969). The reaction is completed by transferring the acyl group to an orthophosphate ion. Bound NAD accelerates the formation and dissociation of the enzyme-G3P complex (Smith and Velick, 1972).

Reductive dephosphorylation also proceeds via an acyl enzyme intermediate; it is formed by the displacement of phosphate from DPGA. The acyl enzyme is then reduced by NADH (Smith and Velick, 1972). NAD is an activator for reductive dephosphorylation (Harrigan and Trentham, 1973; DeVijlders et al., 1970).

Since each monomer of GAPD binds one molecule of NAD or NADH competitively, this binding can affect the structural state of the enzyme and determine the rate of oxidative phosphorylation or reductive dephosphorylation. In the oxidative phosphorylation, the active form of the enzyme is the NAD-GAPD complex, and acylation promotes the release of NADH. According to Velick (Smith and

Velick, 1972), in reductive dephosphorylation the binding of NADH to a vacant active site inhibits the reaction (the inhibition occurs at low DPGA concentrations), but the binding of NADH to an acylated site simply results in reaction. In contrast, the binding of NAD to a vacant site prevents the binding of NADH to that site and promotes acylation at another site; thus NAD accelerates reductive dephosphorylation at low DPGA concentrations. At high DPGA concentrations, bound NAD at the acylated site blocks the binding of NADH and inhibits the reaction.

Other Reactions Catalyzed by Glyceraldehyde 3-P Dehydrogenase. In addition to the basic dehydrogenase reactions discussed above, GAPD catalyzes a number of other reactions--transferase, phosphatase, esterase, diaphorase, transphosphorylase and NADH-NADHX activities. These reactions, their various requirements for the NAD-GAPD complex and for free sulfhydryl groups, and their significance have been summarized by Stancel (Stancel, 1970). Current research still continues on these reactions, their mechanisms and their possible importance in vivo (Francis, Meriwether and Park, 1971; Benitez and Allison, 1973; Foucault et al., 1974).

The arsenolytic reaction, a variation on oxidative phosphorylation, has become the basic assay for GAPD. Arsenate is substituted for phosphate to give an unstable acyl arsenate which is hydrolyzed immediately to the

corresponding acid. This variation permits oxidative phosphorylation to be measured without competition from reductive dephosphorylation.

POSITIVE AND NEGATIVE COOPERATIVITY AND HALF-OF-THE-SITES REACTIVITY. Cooperativity in GAPD is related to pyridine nucleotide binding. Lobster muscle GAPD at 25° binds NAD with negative cooperativity (DeVijlders et al., 1970); rabbit muscle GAPD at 4° and 25° binds NAD (Conway and Koshland, 1968) and NADH (Boers, Oosthuizen and Slater, 1971; Boers and Slater, 1973) with negative cooperativity. Yeast GAPD at 4° and 25° shows positive cooperativity toward binding the second NAD and negative cooperativity toward binding the third and fourth (Cook and Koshland, 1970). Spectroscopic studies at 24° on the molecular basis for negative cooperativity in rabbit muscle GAPD indicate that the cooperativity arises from ligand-induced conformational changes on a previously symmetric molecule (Schlessinger and Levitzki, 1974).

The significance of the above data has been brought into question as a result of investigations at more physiological temperatures. Upon increasing the temperature from 2° to 36°, Velick found the negative cooperativity exhibited by rabbit muscle and liver GAPDs disappeared (Velick, Baggott and Sturtevant, 1970). Also, Jaenicke and Kirschner have independently demonstrated that yeast GAPD at 40° binds NAD with positive

cooperativity (Jaenicke, 1970; Kirschner et al., 1966). Von Ellenrieder has shown that yeast GAPD binds NADH linearly at 25° and 40° (Von Ellenrieder, Kirschner and Schuster, 1972). The question of whether GAPD exhibits negative or positive cooperativity is further complicated by Koshland's recent discovery that binding a single NAD to yeast GAPD can cause positive cooperativity within a subunit (accelerate the acylation and deacylation of its essential thiol) while causing negative cooperativity between subunits (decelerate NAD binding) (Stallcup and Koshland, 1973).

In half-of-the-sites reactivity as exhibited by many multi-subunit enzymes, only half of the subunits can react with active site reagents; yet when these are reacted, all enzymatic activity is lost. Half-of-the-sites reactivity differs from negative cooperativity in that in the former, only half of the subunits can react, while in the latter, all the subunits can be made to react with high enough concentrations of reagents. In contrast, when each subunit reacts independently, it is called linear reactivity.

Work by Stallcup and Koshland (1973) on yeast GAPD has shown that half-of-the-sites reactivity is induced by reacting the essential thiol with almost any sulfhydryl reagent (mercurials, alkylating or acylating agents, disulfides). Once two of the sulfhydryl groups

have been reacted, the remaining two sulfhydryls cannot react and GAPD cannot catalyze any of its enzymatic activities. But reaction of the active site lysines with alkylating and acylating agents permits all the lysines to be reacted and causes GAPD to exhibit linear reactivity toward addition of NAD to the tetramer. In contrast, with rabbit muscle GAPD (Levitzki, 1974) only certain alkylating agents can induce half-of-the-sites reactivity when reacted with the essential thiol.

MOLECULAR STRUCTURE OF GLYCERALDEHYDE 3-P DEHYDROGENASE.

Amino Acid Composition. Of the many GAPDs whose amino acid compositions have been examined, all have four peptide chains of approximately 330 residues (Harris, 1970). The amino acids in these chains are remarkably similar from one species to another: three-fourths of the residues have been conserved through five hundred million years of evolution (Elodi and Liber, 1970). Perhaps the most noteworthy conservations are those of the three tryptophan residues per monomer in all GAPDs (Perham, 1971; Allison and Kaplan, 1964), except rabbit muscle which has four (Harris and Perham, 1963) and Escherichia coli which has eight (Allison and Kaplan, 1964), and the four cysteine residues per monomer in all mammalian GAPDs, except monkey (Perham, 1969) and human muscle (Wolny, 1968) which have three.

In all GAPDs which have been sequenced (lobster muscle [Davidson et al., 1967], pig muscle [Harris and Perham, 1968] and yeast [Jones and Harris, 1972]), the amino acid sequences are remarkably similar; there is 60 per cent homology between the mammalian, yeast and lobster enzymes (Harris, 1970) and a much greater homology of 72 per cent between the mammalian and lobster enzymes (Perham, 1971). The differences in sequence that do exist are highly conservative substitutions that would not significantly disturb the three dimensional structure or mode of action of GAPD (Perham, 1971). Although isozymes of GAPD have been found, evidence for these isozymes having different sequences has not been discovered (Harris, 1970).

Sulfhydryl Groups. Mammalian GAPDs (except monkey and human muscle) have four cysteines per monomer (Perham, 1969), lobster muscle has five (Davidson et al., 1967) and yeast has two (Harris, 1970). The two cysteines conserved are Cys-149, the essential thiol which forms a covalent bond with G3P and DPGA, and Cys-153, a close neighbor of Cys-149 (the numbers refer to the pig muscle sequence) (Harris, 1970). No disulfide bonds are found in native GAPD.

Studies of rabbit, pig and lobster muscle GAPDs using PCMB (Boross, Cseke and Vas, 1969), o-iodobenzoate (Olson and Park, 1964; Ehring and Colowick, 1969),

iodoacetic acid (Perham and Harris, 1963; Park et al., 1961; Harris, Meriwether and Park, 1963; Bernhard and MacQuerrie, 1971), fluorodinitrobenzoate (Shaltiel and Soria, 1969) or DTNB (Wassarman and Major, 1969) show Cys-149 residues react very rapidly. Upon further incubation of pig muscle GAPD with PCMB, Cys-153 residues react at a moderate rate and the remaining cysteines react very slowly (Boross, Cseke and Vas, 1969). But upon further reaction with DTNB, all four remaining cysteines per monomer in lobster muscle GAPD react at an equal rate (Wassarman and Major, 1969). Reaction with either PCMB (Szabolcsi, Biszku and Sajgo, 1960) or DTNB (Wassarman and Major, 1969; Boross, 1969) eventually results in denaturation of GAPD, possibly involving disulfide bond formation and precipitation. Reaction with PCMB also results in the loss of bound NAD (Velick, 1953).

Binding Sites for NAD, NADH, Glyceraldehyde 3-P and 1,3-Diphosphoglycerate. At substrate levels, NADH, G3P and DPGA each have only one binding site per monomer. But whether NAD had one or two binding sites per monomer is an open question; the problem is whether the NADs that are bound to the isolated mammalian GAPDs, that are part of the active NAD-GAPD complex and that show negatively cooperative interactions are also the catalytically active NADs.

The concept of two binding sites comes from studies of activity and negative cooperativity or activity and the NAD-GAPD complex. For example, changes in the amount of NAD bound in an NAD-GAPD complex and changes in the rate of NADH formation by oxidative phosphorylation operate independently (Chance and Park, 1967). This difference indicates that the NAD in the NAD-GAPD complex is not the same as the NAD being reduced to NADH; otherwise, the change in rates would be coordinated. Also, the dissociation constant of the NAD-GAPD complex is different than the dissociation constant for NAD during reductive dephosphorylation (Frieden, 1961); these two dissociation constants should be the same if only a single NAD per monomer is involved in both the enzymatic reaction and the NAD-GAPD complex formation. And lastly, NAD binds to GAPD with negative cooperativity, but oxidative phosphorylation exhibits linear reactivity at every NAD concentration (Peczon and Spivey, 1972).

The concept of a single NAD binding site has arisen more recently. Schlessinger and Levitzki (1974) have strong evidence that the adenine moiety of NAD is solely responsible for the negatively cooperative binding in rabbit muscle GAPD; they argue that the rest of the NAD molecule is then available for catalytic activity and that the activity constants need not reflect the negative cooperativity constants. Duggleby and Dennis

(1974) have concluded that only analogues of NAD which can serve as substrates can form active NAD-GAPD complexes; they believe that identical binding requirements indicate use of the same binding sites. Boers has discovered that the K_m for NAD and the K_i for NADH are compatible with the negatively cooperative binding constants of the less tightly bound NADs; he feels that these NAD binding sites are the catalytically active sites (Boers and Slater, 1973).

Amino Acid Residues in the Active Site. The amino acid residues in the active site include Cys-149, Lys-183, His-38, Cys-281 (Park, 1971), Cys-153 (Moore and Fenselau, 1972), Lys-191 and Lys-212 (Forcina et al., 1971; Zapponi et al., 1973). Cys-149 binds G3P and DPGA (Park, 1971); the sulfhydryl group and the aldehyde carbon form a thioester bond (Racker, 1965). Cys-149 is also involved in the enzyme-coenzyme charge-transfer interaction (Boross and Cseke, 1967; Cseke and Boross, 1967). Lys-183 interacts with the pyrophosphate group on NAD and NADH (Buehner et al., 1973); the adenine moiety is also involved in binding the pyridine nucleotides via its 6-amino group and the 2'-hydroxyl group on the adenine-linked ribose, but the nicotinamide moiety is not involved in binding (Yang and Deal, 1969a, b). His-38 is the nucleophile responsible for removing or donating a hydrogen from or to P_i (Moore and Fenselau, 1972). Either Lys-191 or

Lys-212 is the anion binding site for P_i (Forcina et al., 1971; Zapponi et al., 1973), though Keleti contends that a histidyl residue is the anion binding site (Keleti, 1970). Cys-153 (Moore and Fenselau, 1972) and a different lysyl residue (Foucault et al., 1974) are responsible for maintaining the structure of the active site. Although Cys-281 can accept the acetyl group from Cys-149 at high temperatures in rabbit muscle GAPD (Park, 1971), its importance in the active site is questionable because this residue is missing in the yeast (Perham, 1971) and monkey enzymes (Perham, 1969).

X-ray Crystallographic Studies. Preliminary x-ray diffraction data have been reported for GAPD from lobster muscle (Watson and Banaszak, 1964), crayfish (Campbell et al., 1971), Bacillus stearothermophilus (Suzuki and Harris, 1971) and human muscle (Goryunov et al., 1972). In addition, Watson has published a low resolution electron density map of human skeletal muscle GAPD (Watson, Duee and Mercer, 1972), and Rossman has published a high resolution electron density map of lobster muscle GAPD (Buehner et al., 1974). The structural details described in all cases have been the same, despite the variety of GAPD sources.

Molecules with bound NAD are nearly spherical tetramers with a diameter of approximately 80 Å (Watson, Duee and Mercer, 1972). Molecules without bound NAD may

be less symmetrical than molecules with NAD because the crystals formed without NAD are less symmetrical than the crystals formed with NAD (Goryunov et al., 1972). This change in symmetry correlates well with the conformational changes caused by NAD binding which were observed in spectroscopic studies (Schlessinger and Levitzki, 1974). On the x-ray maps, a loop of electron density forms an integral part of two of the four subunit interfaces and relates the subunits in pairs (Watson, Duee and Mercer, 1972); thus, this provides a structural basis for half-of-the-sites reactivity (Stallcup and Koshland, 1973). The helices are right-handed, while the sheets are all left-handed twists (Chothia, 1973).

The coenzyme binding site, an open conformation associated with a parallel pleated sheet, is nearly identical to the coenzyme binding site of lactate dehydrogenase and very similar to those of a number of other NAD-dependent dehydrogenases. But unlike those of lactate dehydrogenase, the coenzyme binding sites are located close to the subunit interfaces (Buehner et al., 1973), which may explain the observed negative cooperativity (DeVijlders et al., 1970; Conway and Koshland, 1968; Cook and Koshland, 1970).

ISOZYMES OF GLYCERALDEHYDE 3-P DEHYDROGENASE. Isozymes from the Same Tissue. Despite the fact that all chemical and physical studies indicate that the four polypeptide

chains in any tetrameric GAPD molecule are identical, researchers have found isozymes of GAPD in single tissues of turtle, perch, trout, spinach (Lebherz and Rutter, 1967), rabbit (liver and muscle) (Harris, 1970), pig (muscle) (Batke and Cennamo, 1972) and flounder (Marangos and Constantinides, 1974a) and from single strains of yeast (Lebherz and Rutter, 1967; Kirschner and Voigt, 1968; Holland and Westhead, 1973). These isozymes usually exist in sets of five, suggesting the possible combinations of two nonidentical subunits, and are observed by techniques which separate molecules according to charge (electrophoresis, isoelectric focusing, ion exchange chromatography), not according to size or shape. Only a single study has found other differences in a set of isozymes besides the difference in charge used to separate them; the flounder muscle GAPDs differ in sulfhydryl requirement, number of PCMB-reactive groups, heat stability and bound NAD content (Marangos and Constantinides, 1974a). Individual isozymes have not yet been identified as having separate activities or cellular locations from other isozymes.

Isozymes from Different Tissues. Because most enzymes exhibit species-specific differences and because many glycolytic enzymes have tissue-specific isozymes, GAPDs from different species and from different tissues of

the same species have been examined for variation. Remarkably few variations have been found.

Looking at GAPD from various species, the number of differences in amino acid composition and in antigenic determinants increase with the taxonomic distance between species. There are also differences in the amount of NAD bound and the reactivity of the enzymes toward NAD (Allison and Kaplan, 1964; Nagradova and Grozdova, 1974).

Studies of GAPDs from different tissues of the same species have included comparisons of rabbit muscle, liver, brain, heart and kidney enzymes (Bondi, Watkins and Kirtley, 1969; Kochman and Rutter, 1968; Smith and Velick, 1972) and ox muscle and liver enzymes (Lambert and Perham, 1974). No major structural or kinetic differences have been found, although a few minor differences were noted (specifically some chemical differences which Velick and Kirtley consider significant). Smith and Velick (1972) contend that the minor kinetic differences between rabbit muscle and liver GAPDs are sufficient to explain the respective glycolytic and gluconeogenic roles of these enzymes.

DISSOCIATION AND REASSOCIATION OF SUBUNITS. GAPD has been a favorite model for studying the forces binding enzyme subunits together. Such studies use various agents to dissociate and reassociate the enzyme; the process of dissociation is followed by hybridization or by separation

of the dissociated components in sucrose density gradients, by ultracentrifugation or on electrophoresis gels. A list of dissociating agents, their effect on GAPD, the reversal of this effect, the source of the GAPD and the major references is listed in Table 1.

For GAPD, dissociation and inactivation are often linked; in many cases dissociation can cause partial or total inactivation and inactivation eventually brings about dissociation. Likewise, reassociation and reactivation are usually closely linked.

The reassociated and dissociated forms of GAPD are tetramer, dimers, monomers and aggregates. The first three forms are interconvertible; the last is a product of irreversible denaturation. Aggregation is not to be confused with reassociation--reassociation is the formation of an active tetramer; aggregation is a random massing of unfolded monomers which have lost all ability to reassociate.

Dissociation studies have given rise to speculation about the form of the active species of GAPD. From active enzyme sedimentation velocity experiments using rabbit muscle GAPD, Hoagland and Teller have concluded that only the tetramer shows full dehydrogenase activity (Hoagland and Teller, 1969). Jaenicke has drawn similar conclusions from experiments showing that substrate and coenzyme binding shift the dissociation equilibrium for rabbit

Table 1. Dissociation and Reassociation of Glyceraldehyde 3-P Dehydrogenase

Dissociating Agent	Dissociated Form	Requirements for Reassociation	Source[a]	References
PCMB	monomers and dimers	dithiothreitol	R	Smith and Schachman, 1971
low temperature (0-5°)	dimers	20°, phosphate, reducing agent	A	Nagradova and Guseva, 1971
			Y, R	Kirschner and Schuster, 1970
dilution (0.05 mg/ml or less)	monomers and dimers	NAD and phosphate	R	Agatova and Kurganov, 1966
			R	Hoagland and Teller, 1969[b]
			P	Lakatos, Zavodszky and Elodi, 1972
			N	Zavodskii, 1968
			Y	Kirschner and Schuster, 1970
KCN	dimers	not studied	R, P, B	Elodi, 1958
SDS	dimers	not studied	R	Marti and White, 1968
			P	Markovich, Zavodskii and Vol'kenshtein, 1966

Table 1. Continued

Dissociating Agent	Dissociated Form	Requirements for Reassociation	Source[a]	References
urea and guanidine-HCl	monomers	NAD, reducing agent, min ionic strength, low protein concn	R, Y Y F R	Jaenicke, Schmid and Knof, 1968 Deal, 1969 Marangos and Constantinides, 1974b Teipel and Koshland, 1971
ATP (0°)	monomers and dimers	25°, reducing agent (for yeast, sucrose)	Y R P	Stancel and Deal, 1968, 1969 Constantinides and Deal, 1969 Ovadi et al., 1971[b,c]
maleic anhydride	dimers	not studied	P	Ovadi et al., 1971
extreme pH	monomers		R, Y	Jaenicke, 1970
acidic (pH 2)		rapid increase in pH, 0.01 °/o BSA, low protein concn	Y, R	Jaenicke, Schmid and Knof, 1968
basic (pH 11)		dialysis, low protein concn	Y	Shibata and Kronman, 1967
high ionic strength (at least 1 M citrate, phosphate or KCl)	monomers and dimers	reducing agent and warming to 25° or dilution and dialysis against pyrophosphate	R, Y Y C	Jaenicke, 1970 Kirschner and Schuster, 1970 Aune and Timasheff, 1970

Table 1. Continued

Dissociating Agent	Dissociated Form	Requirements for Reassociation	Source[a]	References
$(NH_4)_2SO_4$ and KCl (0.15 M, 0°, 0.1 mg/ml or less)	monomers or dimers	reducing agent and warming to 25°	R	Constantinides and Deal, 1970
			A	Nagradova and Guseva, 1971

[a]The abbreviations used: R = rabbit muscle, Y = yeast, P = pig muscle, B = bovine muscle, A = rat muscle, F = flounder muscle, C = chicken muscle and N = not stated.

[b]Obtained dimers only.

[c]Reassociation not studied.

muscle GAPD from dimer to tetramer (Jaenicke, 1970). However, Ovadi has isolated an active dimer after incubating pig muscle GAPD with ATP and mercaptoethanol for two hours; the dimer is 50 per cent as active as the tetramer (Ovadi et al., 1971). Elodi (1958) and Kirshner and Schuster (1970) have obtained active dimers after dissociating with KCN and high ionic strength respectively.

NAD protects the enzyme against inactivation and dissociation (Hoagland and Teller, 1969; Constantinides and Deal, 1969; Ovadi et al., 1971). This protection presumably comes from NAD maintaining an enzymic structure that is less susceptible to inactivation and dissociation or from NAD competing with the dissociating agent for the same binding site. NAD is also required for reactivation from urea, guanidine-HCl or dilute solution. Based on the requirements for NAD for in vitro reassembly, it has been speculated that NAD may be required for the in vivo folding of newly synthesized GAPD polypeptide chains and their association into tetramers (Deal, 1970; Teipel and Koshland, 1971).

EFFECTS OF ATP. ATP, a well-known effector of various glycolytic enzymic activities and a partial structural analog of NAD, has three negative effects on GAPD activity (Stancel and Deal, 1968): (1) ATP produces an immediate inhibition by competing with NAD for the

NAD binding sites (Yang and Deal, 1969a), (2) ATP causes a time-dependent inactivation by dissociating the enzyme into subunits (Constantinides and Deal, 1969; Stancel and Deal, 1968, 1969), and (3) ATP stimulates a very rapid loss of activity in the presence of chymotrypsin by causing structural changes which make the enzyme more susceptible to proteolytic degradation (Yang and Deal, 1969b).

Immediate Inhibition. The immediate inhibition by ATP of oxidative phosphorylation was first studied with yeast GAPD at 25° (Yang and Deal, 1969a). Besides ATP, AMP, ADP and cAMP were found to be inhibitory; all the compounds showed competitive inhibition with respect to NAD. From this competition and a comparison of the structure of ATP and NAD, it was concluded that ATP and NAD compete for the same binding site (Yang and Deal, 1969a, b). Park's laboratory extended these studies to rabbit muscle GAPD using physiological concentrations of substrates and effectors. Their results were similar to those of Yang and Deal, except they found ATP to also be competitive with respect to P_i. From this additional competition, they concluded that the ATP-binding site covered the P_i-binding site as well as the NAD-binding site (Oguchi, Meriwether and Park, 1973). Adenine nucleotide inhibition has also been observed by Sapag-Hagar (1969) who used ATP, ADP and AMP to inhibit rabbit

muscle GAPD and by Nagradova, Vorona and Asriyants (1969) who used ADP and AMP to inhibit yeast and rabbit muscle and heart GAPDs.

Park's laboratory has also studied the ATP-inhibition of the "nonphysiological" reactions of GAPD (reactions other than oxidative phosphorylation and reductive dephosphorylation). They have found three points where adenine nucleotides can inhibit the reactions: coenzyme binding, formation of enzyme-substrate complexes and transfer of S-acyl group from active site to acceptors (Francis, Meriwether and Park, 1971).

Time-Dependent Inactivation and Dissociation. The time-dependent inactivation caused by the dissociation of GAPD in the presence of ATP was first discovered in Deal's laboratory (Stancel and Deal, 1968). Using yeast GAPD (Stancel and Deal, 1969), the optimal conditions for inactivation and dissociation into monomers were 1-2 mM ATP, 0°, 0.03-0.1 mg/ml GAPD, pH 9.0 and 0.1 M mercaptoethanol. AMP and cAMP did not dissociate the enzyme. The dissociation could be partially prevented by AMP, cAMP or any of the substrates and could be reversed by warming to 17°. The optimal conditions for reversal were 0.04 mg/ml GAPD, pH 7.0, 1-2 mM ATP, 17°, 10 per cent sucrose and 0.1 M mercaptoethanol. Extending the studies to rabbit muscle GAPD, Deal's

laboratory found a similar, yet different dissociation (Constantinides and Deal, 1969). The process still required 1-2 mM ATP, 0.1 M mercaptoethanol and 0°, but it occurred much more rapidly (in less than one hour instead of taking 12-14 hr), was pH independent, happened at higher GAPD concentrations and resulted in dimers at high protein concentrations and monomers at low protein concentrations. This dissociation was prevented by 1 M $(NH_4)_2SO_4$ or 2 mM NAD. The reversal could still be accomplished by warming the enzyme, but optimal conditions were 23°, no ATP and no sucrose. Feeling that 0° was not physiological, Park's laboratory attempted the inactivation experiments with rabbit muscle GAPD at 25° (Oguchi, Meriwether and Park, 1973). They were able to obtain results similar to those of Constantinides and Deal if they increased the ATP concentration to 6 mM. They did not study the dissociation or reactivation. In addition they demonstrated that the immediate inhibition by ATP and the time-dependent inactivation were two separate processes.

There have been several other studies on the dissociation of GAPD by ATP. Keleti's laboratory dissociated pig muscle GAPD with ATP at 0° into a dimer which was active in the presence of mercaptoethanol. They demonstrated that four moles of ATP were bound per tetramer and that the dissociation caused the exposure

of a normally buried sulfhydryl group which, in the absence of mercaptoethanol, could form a disulfide bond with the same residue on the neighboring monomer (Ovadi et al., 1971; Ovadi, Nuridsany and Keleti, 1973). Lebherz, Savage and Abacherli (1973) demonstrated an ATP-mediated subunit exchange between isozymes of trout skeletal muscle GAPD at 25°. In addition to the subunit exchange, ATP caused a time-dependent inactivation which they did not characterize. Both the exchange and the inactivation were prevented by NAD. Darnall and Murray (1972) studied dissociation by ATP of rabbit muscle and human erythrocyte GAPDs using the physiological conditions for the human erythrocyte. Dissociation at 25° required a mixture of ATP and cacodylic acid and was reversed by 2,3-diphosphoglycerate. Despite examination of glycolytic intermediates, tricarboxylic acid intermediates and several amino acids, they could not find a physiological compound that would replace cacodylic acid in their dissociation system.

Stimulation of Proteolytic Digestion. The ATP-stimulated loss of enzymic activity in the presence of chymotrypsin has been studied only by Yang and Deal (1969b). They hoped to gain some insight into the mechanism of ATP binding and into the control of enzyme degradation. ATP, and to a lesser extent ADP and AMP, alter the structure of yeast GAPD in a manner which encourages

its digestion by chymotrypsin. This ATP effect is prevented by NAD and cAMP. The destabilization seems to be a function of the number and the charge of the terminal phosphate groups of the adenine nucleotide.

It is interesting that the binding of 2,4,6-trinitrobenzenesulfonic acid (Foucault et al., 1974) and the removal of bound NADs (Cantau, Jaureguiberry and Pudles, 1970; Jaenicke, 1970; Fenselau, 1972) also increase the susceptibility of rabbit muscle GAPD to digestion by chymotrypsin. Since NAD binds competitively with ATP (Yang and Deal, 1969a, b) and 2,4,6-trinitrobenzenesulfonic acid binds competitively with NAD (Foucault et al., 1974), it seems likely that NAD, ATP and 2,4,6-trinitrobenzenesulfonic acid all cause the same structural alteration.

EVIDENCE FOR AND AGAINST THE BINDING OF ZINC TO GLYCERALDEHYDE 3-P DEHYDROGENASE. Zinc is a functional component of several dehydrogenases, such as alcohol (Vallee and Hoch, 1955), malic (Harrison, 1963) and glutamic dehydrogenases (Adelstein and Vallee, 1958). Because zinc stabilizes the structure and participates in the catalytic function of these enzymes by being involved in NAD binding, it has been proposed that zinc might also be present in other pyridine nucleotide dependent dehydrogenases (Vallee, 1960).

By emission spectrochemistry and analysis for metal content, Vallee found metal ions, presumably zinc, in yeast and rabbit muscle GAPDs (Vallee et al., 1956). Subsequently, Keleti reported that pig muscle, cow muscle, crayfish muscle and yeast GAPDs contained two to three moles of zinc per mole of enzyme. Further studies indicated that removal of zinc from the enzyme led to protein denaturation and that chelation of zinc led to inhibition of enzymic activity; from these results Keleti proposed that zinc was essential for the catalytic activity and the maintenance of steric structure in GAPD (Keleti et al., 1962; Keleti and Telegdi, 1959a,b). Keleti has also studied the exchangability of zinc on various GAPDs (Keleti, 1964, 1966), and Polgar has studied the amino acid residues of GAPD and the areas of its substrates that bind zinc (Polgar, 1964).

In opposition to this data, Ferdinand and Park, working independently, have not found significant amounts of zinc bound to rabbit muscle, pig muscle or yeast GAPDs (Ferdinand, 1964; Barkman, Sandstead and Park, 1970). Park has further shown that zinc is not required for coenzyme binding, substrate binding or catalytic activity (Barkman, Sandstead and Park, 1970). Other workers have demonstrated that GAPD is actually inhibited by heavy metals (Velick and Furfine, 1963) and that its activity is increased in the presence of chelating agents (Nagradova, 1965).

PHYSICAL AND CHEMICAL PROPERTIES OF PIG MUSCLE AND PIG LIVER GLYCERALDEHYDE 3-P DEHYDROGENASES. Pig muscle GAPD can be purified by two methods (Elodi and Szorenyi, 1956; Harrigan and Trentham, 1973). Pig liver GAPD has been purified by only one method (Dagher and Deal, in preparation). Crystalline pig muscle GAPD has four molecules of NAD bound per tetramer (Ovadi et al., 1971; Harrigan and Trentham, 1973); crystalline pig liver GAPD has two or three molecules of NAD bound per tetramer (Dagher and Deal, in preparation). The isoelectric point for the pig liver enzyme is 8.8 (Dagher and Deal, in preparation); the isoelectric point for the pig muscle enzyme is 8.1 to 8.6 (Batke and Cennamo, 1972).

The specific activity is 150 to 220 μmoles/min/mg for both purified enzymes, and their pH optimums for oxidative phosphorylation are between 8 and 9. Kinetic constants for the pig liver enzyme include a K_m for NAD of 12 μM and a K_m for G3P of 240 μM in oxidative phosphorylation and a K_m for NADH of 23 μM in reductive dephosphorylation. Kinetic constants for pig muscle GAPD are similar to those for lobster muscle GAPD; approximate comparative values are estimated to be a K_m for NAD of 13 μM, a K_m for G3P of 90 μM and a K_m for NADH of 3.3 μM. With both enzymes, NAD is a competitive inhibitor of NADH in reductive dephosphorylation and exhibits a K_i of 850 μM for pig liver GAPD and of

300 μM for pig muscle GAPD (Harrigan and Trentham, 1973; Velick and Furfine, 1963; Dagher and Deal, in preparation).

Pig muscle GAPD has a molecular weight of 145,000 ± 6000 for its tetramer and 36,000 ± 1500 for its monomer (Harrington and Karr, 1965). Pig liver GAPD has a molecular weight of 148,000 for its tetramer and 38,000 for its monomer (Dagher and Deal, in preparation). A partial specific volume of 0.737 ml/g at 20° has been measured for the pig muscle enzyme (Elodi, 1958). The amino acid composition and sequence of pig muscle GAPD have been determined (Harris and Perham, 1968), and the reactivity of its sulfhydryl groups has been studied (Boross, Cseke and Vas, 1969). The sulfhydryl residues can be divided into three classes based on their reactivity: Cys-149 is very reactive, Cys-153 is moderately reactive and Cys-244 and -281 do not react unless the subunits are unfolded.

A number of measurements indicate that pig muscle GAPD has a compact hydrophobic core amounting to 40 per cent of its molecular weight and a 28 to 48 per cent α-helix content (Zavodszky, Abaturov and Varshavskii, 1966; Markovich, Zavodskii and Vol'kenshtein, 1966). These conclusions are based on the following evidence. ORD spectra for pig muscle GAPD with and without NAD and NADH and in the presence of SDS have been measured.

Using the parameters λ_c, b_o, a_o, K_c, H_{193} and H_{225}, the NAD-free enzyme is 34 per cent ordered structure; the NAD-GAPD complex is 43 per cent ordered structure. The dispersion constants, λ_c and b_o, decrease 4 per cent on the addition of NADH to the NAD-free enzyme and decrease 5 per cent on the addition of SDS. Infrared spectrophotometric studies of hydrogen-deuterium exchange indicate that 32 per cent of the peptide hydrogens are inaccessible to water, 15 per cent are accessible within a measurable time span and 53 per cent exchange immediately. Addition of NAD or NADH increases the amount of inaccessible peptide hydrogens by 10 and 4 per cent respectively. The viscosity of native GAPD is 0.033 dl/g and of GAPD in SDS is 0.088 dl/g.

Pig muscle GAPD exists as five isozymes which can be separated by isoelectric focusing (Batke and Cennamo, 1972). Smith and Velick (1972) claim that such isozymes arise from differential binding of NAD, but the pig muscle isozymes all contain the same amount of NAD. The enzyme can be dissociated into dimers and/or monomers by KCN (Elodi, 1958), dilution (Lakatos, Zavodszky and Elodi, 1972) and ATP (Ovadi et al., 1971; Ovadi, Nuridsany and Keleti, 1973). Some preliminary studies have indicated that pig liver GAPD could be dissociated by ATP (Dagher and Deal, in preparation). One of the objectives of this thesis was to define this phenomenon in more detail.

According to Keleti, pig muscle GAPD contains two or three moles of zinc per mole of enzyme which are essential for catalytic activity and for maintenance of the steric structure (Keleti et al., 1962; Keleti and Telegdi, 1959a,b). However, Barkman, Sandstead and Park (1970) contend that there is no significant amount of zinc in pig muscle GAPD and that zinc is not required for catalytic activity; still, it is interesting to note that what they call an insignificant quantity of zinc in pig muscle GAPD (0.115 to 0.146 moles of zinc per mole of enzyme) is three to five fold higher than the amount they found in rabbit muscle or yeast GAPD.

MATERIALS AND METHODS

REAGENTS. Fresh pig livers were obtained from the Peet Packing Co., Chensaning, Michigan, and frozen within 15 min of slaughter. NAD and ATP (disodium salt) were purchased from P-L Biochemicals, Inc. Cysteine, tannic acid, gum arabic, ATP (disodium salt), BSA and DTNB were products of Sigma Chemical Co. Lactate dehydrogenase (dog muscle) was purchased from Boehringer Mannheim Corp. G3P was obtained from Sigma Chemical Co. as the diethylacetal barium salt and converted into the free acid as previously described (Deal, 1969). Imidazole was purchased from Sigma Chemical Co. and was recrystallized once from chloroform:ether. DEAE-Sephadex A-50, SE-Sephadex C-25 and coarse Sephadex G-25 were products of Pharmacia Fine Chemicals. Norit A was obtained from Fischer Scientific Co. and was activated by acid-base washing (Heppel, 1963; Smith and Khorana, 1963).

ENZYME PREPARATION. GAPD was crystallized from pig liver by adapting to a large scale the method of Dagher and Deal (in preparation).

A whole pig liver (1100-1300 g) was thawed for 24 hr at 4°. The liver was cut into large pieces and

homogenized for one minute in a Waring blender with 1100-1300 ml cold 10 mM $ZnCl_2$ and 25 mM mercaptoethanol. The homogenate was centrifuged at 13,800 x g for 30 min. Its supernatant was collected and made 50 mM in EDTA and 50 mM in mercaptoethanol.

An ammonium sulfate fractionation at 0° was performed on the cold supernatant solution. The precipitate which formed between 65 and 95 per cent saturation (calculated from Warburg's formula [Warburg and Christian, 1939]) was collected by centrifugation at 27,300 x g for 30 min and dissolved in 40 ml 10 mM imidazole pH 7.5, 25 mM mercaptoethanol. The resuspended pellet was divided into two batches and desalted on a column of coarse Sephadex G-25 (6 x 96 cm) equilibrated in distilled deionized water. The salt-free eluates were combined, made 50 mM in mercaptoethanol and concentrated to 40 ml in an Amicon TCF 10 ultrafiltration cell using a PM-30 membrane.

The concentrated solution was made 25 mM in mercaptoethanol and adjusted slowly to pH 7.3 with 0.1 N NH_4OH. The precipitate which formed was removed by centrifugation at 39,100 x g for 5 min. The supernatant was chromatographed on a column of DEAE-Sephadex A-50 (6 x 44 cm) equilibrated in 10 mM imidazole pH 7.3, 25 mM mercaptoethanol. The enzyme was in the first protein peak washed from the column by the buffer. This

peak was collected, made 25 mM in mercaptoethanol and concentrated to 20 ml in an Amicon TCF 10 ultrafiltration cell using a PM-30 membrane.

The concentrated solution was made 25 mM in mercaptoethanol, 5 mM in EDTA and 0.2 mM in NAD and adjusted slowly to pH 6.6 with 0.1 N acetic acid. The precipitate which formed was removed by centrifugation at 39,100 x g for 5 min. The supernatant was applied to a column of SE-Sephadex C-25 (2.5 x 22 cm) equilibrated in 10 mM imidazole, 5 mM EDTA pH 6.6, 25 mM mercaptoethanol. The enzyme was eluted with a linear gradient of 0 to 0.3 M KCl, using 300 ml of each solution. The majority of the enzyme appeared in the last protein peak off the column. This peak was collected, made 25 mM in mercaptoethanol and concentrated to 7 ml in an Amicon 52 ultrafiltration cell using a PM-30 membrane.

The concentrated solution was subjected to the crystallization procedure of Jakoby (1971) using 2 ml solutions of various $(NH_4)_2SO_4$ concentrations containing 5 mM mercaptoethanol, 0.2 mM NAD and 50 mM Tris-HCl pH 8.4, instead of 1 ml solutions. The series of per cent saturation in $(NH_4)_2SO_4$ used was 91, 88, 85, 82, 78, 75, 72, 69, 66, 63 and 60 per cent.

The yield was 30 mg of crystalline GAPD with a specific activity of 160 to 220 μmoles/min/mg. It could be stored in its crystallization liquor at a

concentration of greater than 1 mg/ml for at least four months at 4° without loss of activity. The crystalline enzyme gave a single band on SDS polyacrylamide gels (method of Fairbanks, Steck and Wallach [1971] as modified by Welton [1974]).

ENZYME ASSAY. Oxidative phosphorylation was measured at 25° in a Beckman DU monochromator equipped with a Gilford multiple sample absorbance recorder. The assay was the "low NAD-G3P assay" of Deal (1969), except the final concentrations of NAD, G3P and GAPD were 2.5 mM, 0.7 mM and 0.25 μg/ml respectively and Tris-HCl pH 8.5 was substituted for glycylglycine. The concentrations of the G3P and NAD stocks were measured spectrophotometrically by the method of Dagher and Deal (in preparation). An enzyme unit was defined as the amount of enzyme reducing one μmole of NAD per minute at 25°.

PROTEIN DETERMINATION. Protein was determined by the method of Mejbaum-Katzenellenbogen and Dobryszyka (1959). The tannin reagent was routinely warmed and filtered before each use. The 20 min incubation period was employed. A standard curve using crystalline BSA was constructed for each experiment. Turbidity was measured at 650 nm.

A modification of this procedure was used to measure protein on sucrose gradients. Tannin reagent, 0.1 ml, was added to each 0.1 ml sucrose fraction, and

the mixtures were incubated for 20 min at 25°. Gum arabic, 0.2 ml, was added, and the turbidity was measured at 650 nm. This method gave a relative protein concentration which could not be translated into mg/ml because sucrose and the various buffers used in the gradients affected the turbidity and the numerous standard protein solutions required for quantitation were not designed.

INACTIVATION, DISSOCIATION, REACTIVATION AND REASSOCIATION PROCEDURES. For routine handling, a slurry of GAPD crystals was centrifuged at 34,800 x g for 10 min, and the pellet was resuspended in 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol. The resuspended enzyme was dialyzed against 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol for 12 to 18 hr at 4°.

To inactivate the enzyme, GAPD was diluted into the standard ATP inactivation buffer which consisted of 50 mM imidazole, 0.1 M EDTA pH 7.5 containing 80 mM mercaptoethanol and 10 mM ATP to a protein concentration of 0.1 mg/ml and incubated at 0°. These conditions were always kept constant except for the variable under examination. The inactivation was followed by periodic assaying and usually required 10-12 hr.

During inactivation, dissociation occurred. Dissociation was followed by sucrose density gradient centrifugation according to the procedure of Martin and

Ames (1961) as described by Constantinides and Deal (1969) with the following exceptions. The 5-20 per cent sucrose gradients were in 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol, 10 mM ATP (the standard ATP inactivation buffer). These conditions could be slightly varied to reflect more accurately the inactivation conditions. An indirect marker of 0.1 mg/ml pig liver GAPD (7.8 S) or 0.125 mg/ml dog muscle lactate dehydrogenase (7.45 S) was used; it is called an indirect marker because the marker enzyme is not in the same gradient tube as the sample (and cannot be because the enzyme was located by protein determination as well as activity determination). The indirect marker was always run without ATP. A 0.25 ml sample was layered on each gradient; the large volume was chosen to minimize dilution. The gradients were centrifuged at 45,000 rpm for 13.5 hr at 0° in a Beckman Model L3-50 with a SW 50.1 rotor. Ten drop fractions were collected; they had a volume of approximately 0.1 ml. Each gradient tube yielded 34 to 37 fractions. The fractions were assayed before and after warming to 25°. The fractions were also analyzed for protein.

To reactivate the enzyme, the inactivated GAPD (incubated at 0° for 10 to 12 hr) was warmed to 25° for 5 min. Any compounds which were added to aid the

reactivation were put in the inactivated sample before the warming started. The reactivation was measured by assaying.

Reassociation of the reactivated enzyme was followed by sucrose density gradient centrifugation as described above. The samples were centrifuged at 45,000 rpm for 7.75 hr at 25°.

AMINO ACID ANALYSIS. To prepare the enzyme for analysis, the bound NAD was removed by charcoal treatment. A mixture of 14 mg activated Norit A and 1.2 mg GAPD in 0.6 ml crystallization liquor was centrifuged to remove the charcoal and NAD. The OD_{280}:OD_{260} of the supernatant was 1.36, indicating that a little NAD still remained bound to the enzyme (Fox and Dandliker, 1956). It was later learned that more of the NAD could probably have been removed by incubating the activated Norit A-GAPD mixture for a longer period of time (Ovadi et al., 1971).

The NAD-free enzyme was dialyzed against distilled deionized water for 24 hr at 25°. The dialyzed enzyme was concentrated to 1.0 mg/ml on a rotary evaporator. Equal volumes of concentrated enzyme and 12 N HCl were combined, sealed into vials and heated at 110° for 18 and 56 hr to hydrolyze. After hydrolysis, the solutions were evaporated to dryness, and the residues resuspended in 0.2 M citrate buffer, pH 2.2. The analysis was performed on a modified Technicon amino acid analyzer

with a computerized integrator. The concentration of each amino acid was calculated by comparison to an internal standard, norleucine.

The total number of cysteine residues was determined by DTNB titration in SDS (Habeeb, 1972). The total number of trytophan residues was determined by the spectrophotometric method of Edelhoch (1967), using SDS instead of guanidine-HCl. The protein concentration of the samples had to be determined before the SDS was added.

The partial specific volume was determined from the amino acid composition by the method of Cohn and Edsall (1943) as described by Kayne (1966).

DTNB REACTION. The reactivity of the sulfhydryl groups in native GAPD was determined by DTNB titration (Habeeb, 1972). The reaction was measured in a Cary 15 recording spectrophotometer. The reaction mixture contained 240 µg GAPD, 200 µg EDTA and 40 µg DTNB (65 fold excess compared to GAPD) in 0.08 M sodium phosphate buffer, pH 8.0. The number of sulfhydryl residues titrated per tetramer was calculated with the molar extinction coefficient of 13,600 at 412 nm.

RESULTS

INACTIVATION AND DISSOCIATION OF PIG LIVER GLYCERALDEHYDE 3-P DEHYDROGENASE. The ability of ATP to inactivate and dissociate yeast (Stancel and Deal, 1968, 1969), rabbit muscle (Constantinides and Deal, 1969) and pig muscle GAPDs (Ovadi et al., 1971) at 0° has been established. Experiments by Dr. Dagher of this laboratory indicated that pig liver GAPD can also be inactivated and dissociated by ATP at 0° (Dagher and Deal, in preparation). Dr. Dagher followed the dissociation by sucrose density gradient centrifugation, analyzing the gradients for dissociated enzyme by first warming the fractions to reactivate the enzyme and then assaying each for activity (Constantinides and Deal, 1969; Stancel and Deal, 1969). Using this technique he had great difficulties obtaining reproducible results. This problem stemmed from the low activity of GAPD under these conditions and the inability of the dissociated enzyme to reactivate.

The first part of this thesis work was designed to clarify and extend these preliminary results. Experiments indicated that GAPD is more readily inactivated and dissociated (by dilution) when 0.1 M EDTA is present.

Investigations of inactivation and dissociation by ATP at 0° showed that this process could also occur in the presence of 0.1 M EDTA. Furthermore, the native GAPD retained most of its activity in the presence of 0.1 M EDTA, and the dissociated enzyme could be reactivated under these conditions. Hence, the next logical step was to characterize the dissociation and inactivation of pig liver GAPD by ATP at 0° in the presence of 0.1 M EDTA.

Unless otherwise indicated, the standard conditions chosen for inactivation and dissociation were (1) 0.1 M EDTA, (2) 0.1 mg/ml GAPD, (3) pH 7.5, (4) 10 mM ATP, (5) 80 mM mercaptoethanol, and (6) 0°. The enzyme was routinely incubated under these conditions for 10 hr. Inactivation and dissociation were followed as described in Materials and Methods. The gradients used to study dissociation contained 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol and 10 mM ATP. The fractions from the gradients were analyzed for both protein and GAPD activity so that inactive, as well as active, enzyme could be located.

Activity determination involved assaying the fractions before and after warming to 25°. The recovery of activity from the gradients was generally poor, especially in the presence of ATP. For control samples at 0° (no ATP), 40 to 60 per cent of the activity applied was recovered; for standard samples (see above paragraph),

11 to 27 per cent of the activity applied was recovered; and for other samples under nonoptimal conditions, 0 to 10 per cent of the activity applied was recovered. The poor recovery could have been caused by the presence of sucrose, since sucrose decreased reactivation (see section "Reactivation and Reassociation of Glyceraldehyde 3-P Dehydrogenase"). Warming the fractions to 25° increased the recovery of activity by 1.2 to 3.2 fold in the gradients with ATP, but did not increase recovery in gradients without ATP.

Effect of Incubation Time. When GAPD was incubated under the standard inactivation conditions for various periods of time, it lost activity as shown in Figure 2. Virtually all of the loss occurred during the first 12 hr, after which the activity tended to remain the same. GAPD incubated under similar conditions without ATP also showed some loss of activity, but it was small compared to the loss in the presence of ATP.

Inactivation was accompanied by dissociation as shown by sucrose density sedimentation velocity experiments (Figure 3). Native pig liver GAPD had a sedimentation coefficient of $S_{20,w}$ = 7.8 S (lactate dehydrogenase as a marker), while maximally inactivated enzyme had a sedimentation coefficient of approximately 5.0 S. In the absence of ATP, the sedimentation coefficient of GAPD did not change with incubation at 0° for at

Figure 2. Effect of incubation time at 0° on inactivation of pig liver GAPD by ATP. Samples, prepared from stock crystals as described in Materials and Methods, were incubated in the standard ATP inactivation buffer (see Materials and Methods) for the times indicated. A control (no ATP) was treated in exactly the same way as the test samples. All values were corrected to the same original specific activity (before incubation). Most of the inactivation occurs after 10-12 hr exposure. Therefore, 10 hr was adopted as the standard time of incubation for most of the following studies, unless indicated otherwise. The filled symbols represent samples exposed to ATP, while the open symbols represent controls in the absence of ATP.

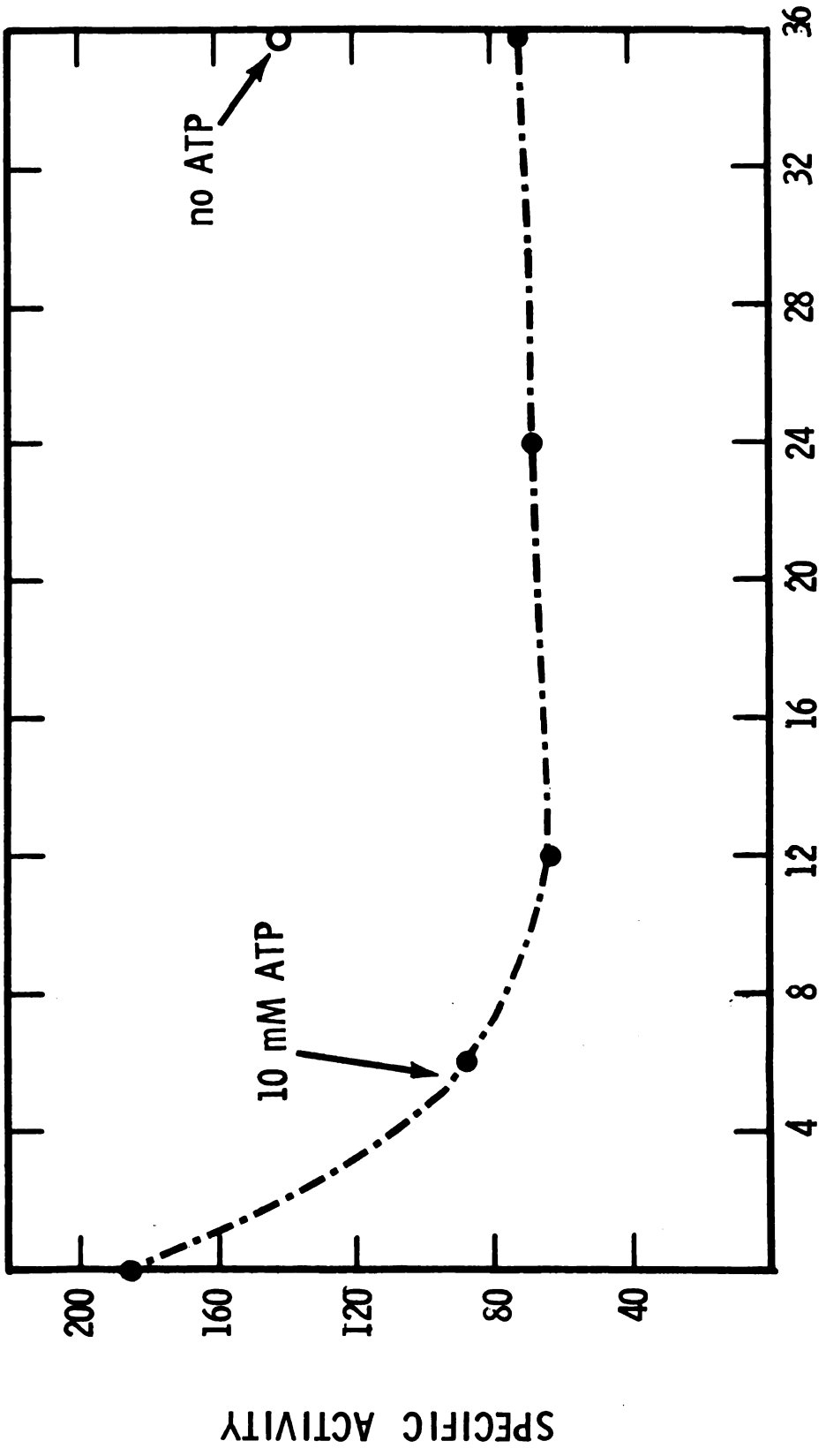
SPECIFIC ACTIVITY
200
160
120
80
40
TIME (HOURS)
4
8
12
16
20
24
28
32
36
10 mM ATP
no ATP

Figure 2

Figure 3. Effect of incubation time at 0° on dissociation of pig liver GAPD by ATP. These studies were carried out on the enzyme samples described in the legend for Figure 2. The samples were centrifuged in 5-20 °/₀ sucrose gradients at 45,000 rpm for 13.5 hr at 0° (see Materials and Methods). The composition of each gradient tube was the same as the corresponding inactivation solution, except for the added sucrose. An indirect marker of pig liver GAPD was run in a gradient tube containing sucrose and the standard ATP inactivation buffer minus ATP; it is called an indirect marker because the marker enzyme is not in the same gradient tube as the sample (and cannot be because the enzyme was located by protein determination). The marker enzyme was in addition to the control without ATP (and also in addition to other controls run in certain later experiments which will be indicated as needed). The positions of the enzyme peaks in the gradients were located by activity and protein determination. Activity was determined before and after incubation at 25°; the incubation at 25° generally reactivated much of the inactive enzyme. The sedimentation coefficients indicated are those calculated from the protein determination. The times indicated are the times prior to centrifugation and do not include the time of centrifugation. The control (no ATP) had a sedimentation coefficient of $S_{20,w}$ = 7.8 S (not plotted). The sedimentation coefficient at 0 hr may be low (see text).

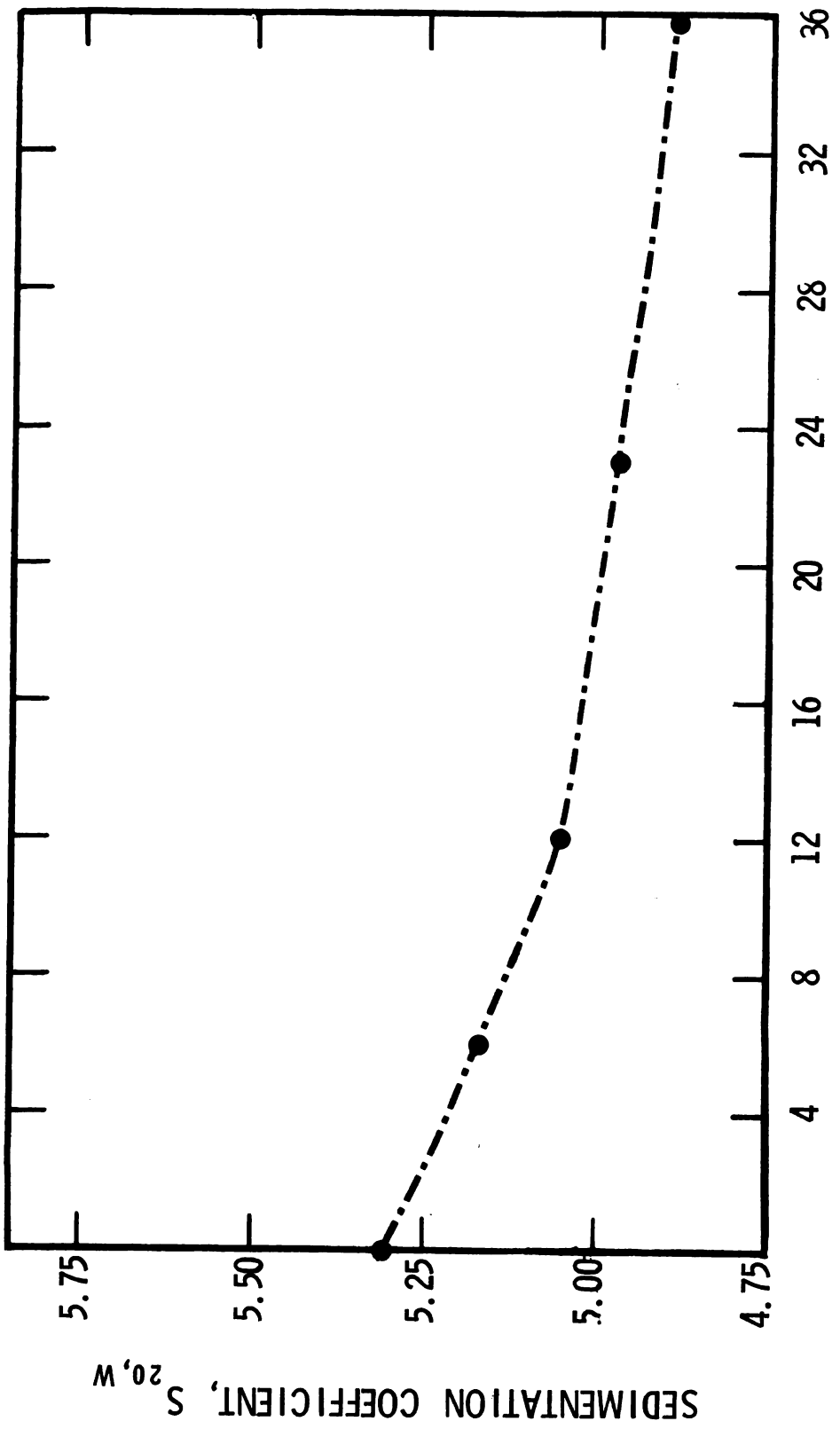
SEDIMENTATION COEFFICIENT, $S_{20,w}$
5.75
5.50
5.25
5.00
4.75
4
8
12
16
20
24
28
32
36
HOURS INCUBATED BEFORE BEGINNING SEDIMENTATION

Figure 3

least 36 hr, although slight inactivation did occur. In the presence of ATP, the enzyme inactivated and dissociated rapidly during the first 12 hr; after that time, it did not inactivate further, but continued to dissociate slowly. The sedimentation coefficient in ATP at 0° for 0 hr is probably low; presumably the enzyme was dissociating during the 10 hours of centrifugation in the gradient run under dissociation conditions (ATP, 0°).

A typical sedimentation pattern for native and ATP-dissociated GAPD is shown in Figure 4. The nearly exact correspondence between the location of the enzyme peaks as determined by protein analysis and the location as determined by activity assays is clearly evident. The dissociated enzyme was still active after centrifugation, but had much less activity than the native enzyme.

Effect of EDTA. EDTA partially protected GAPD against inactivation (Figure 5), but not dissociation, in ATP solutions at 0°. After 10 hr in solutions containing both ATP and EDTA, GAPD lost 50 per cent of its activity, while in solutions containing ATP and no EDTA, the enzyme lost 82 per cent of its activity. And from the gradient tube containing ATP and no EDTA, no activity was recovered. EDTA also protected GAPD against inactivation in the absence of ATP. From the gradient tube containing EDTA and no ATP, 71 per cent of the activity applied was

Figure 4. Typical sedimentation patterns for native and ATP-dissociated pig liver GAPD. The dissociation sample and the native control were prepared and treated as described in the legend for Figure 2; the incubation time was 10 hr. The samples were then centrifuged in sucrose density gradients as described in the legend for Figure 3. The activity plotted is that measured at 4°. In this figure, as in all the following figures of inactivation curves and sedimentation patterns for dissociated enzyme (except the graphs of native GAPD in Figures 7 and 10), a solid dot represents 0.1 mg/ml GAPD inactivated and dissociated in the standard ATP inactivation buffer, and an open symbol represents a control sample (not to be confused with the marker samples which are never plotted).

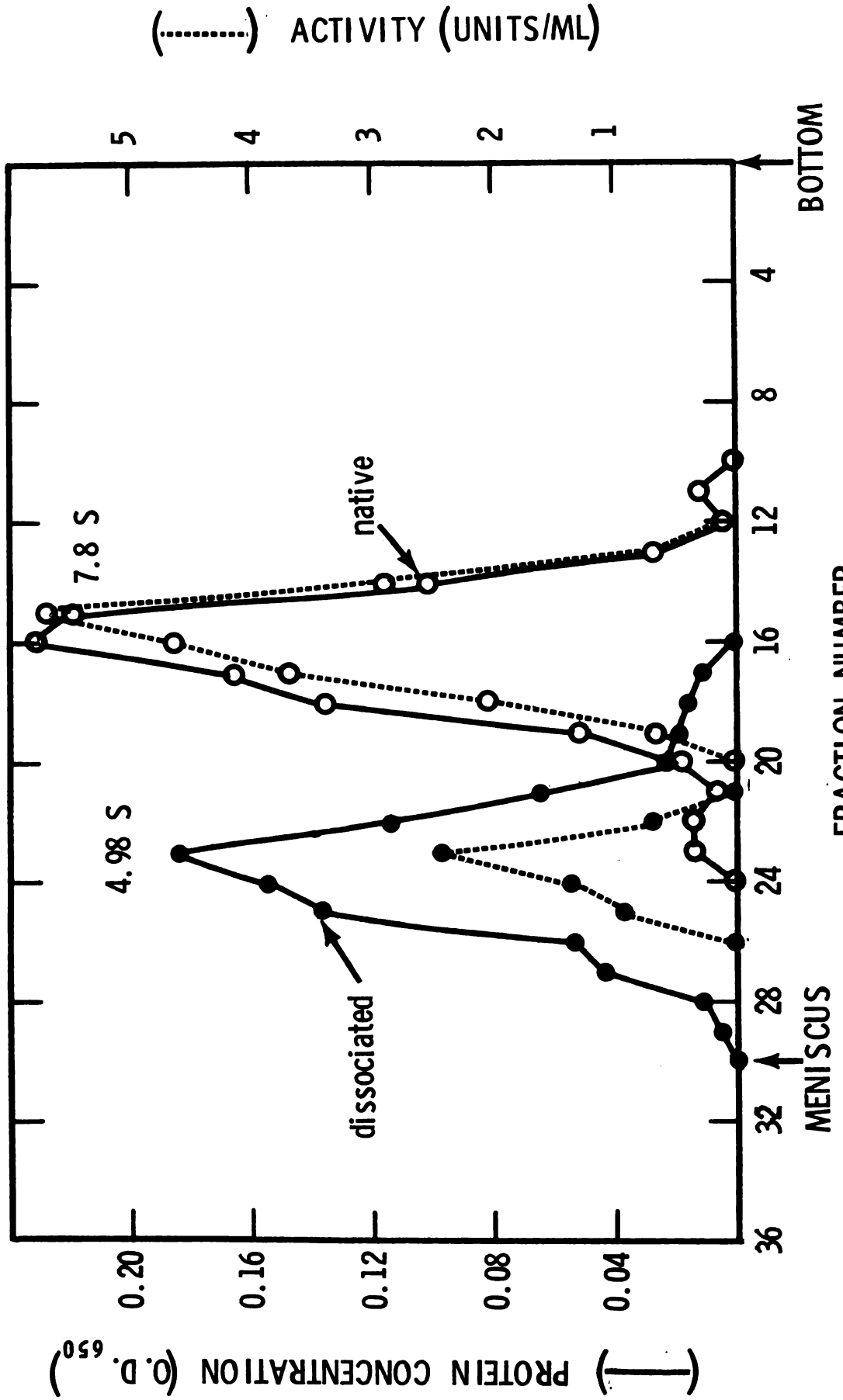
(.....) ACTIVITY (UNITS/ML)
5
4
3
2
1
BOTTOM
native
7.8 S
4.98 S
dissociated
0.20
0.16
0.12
0.08
0.04
4
8
12
16
20
24
28
32
36
MENISCUS
FRACTION NUMBER
(——) PROTEIN CONCENTRATION (O.D.650)

Figure 4

Figure 5. Effect of EDTA on inactivation of pig liver GAPD by ATP at 0°. Dissociation samples and controls were prepared and treated as described in the legend for Figure 2, except EDTA was omitted from some of them. The sedimentation coefficients measured for these enzyme solutions (obtained by sucrose density gradient centrifugation as described in the legend for Figure 3) are presented in the text. (The samples without EDTA had an indirect marker [see Figure 3] of dog muscle lactate dehydrogenase because the gradients without EDTA were less dense and pig liver GAPD is not as active in sucrose without EDTA.)

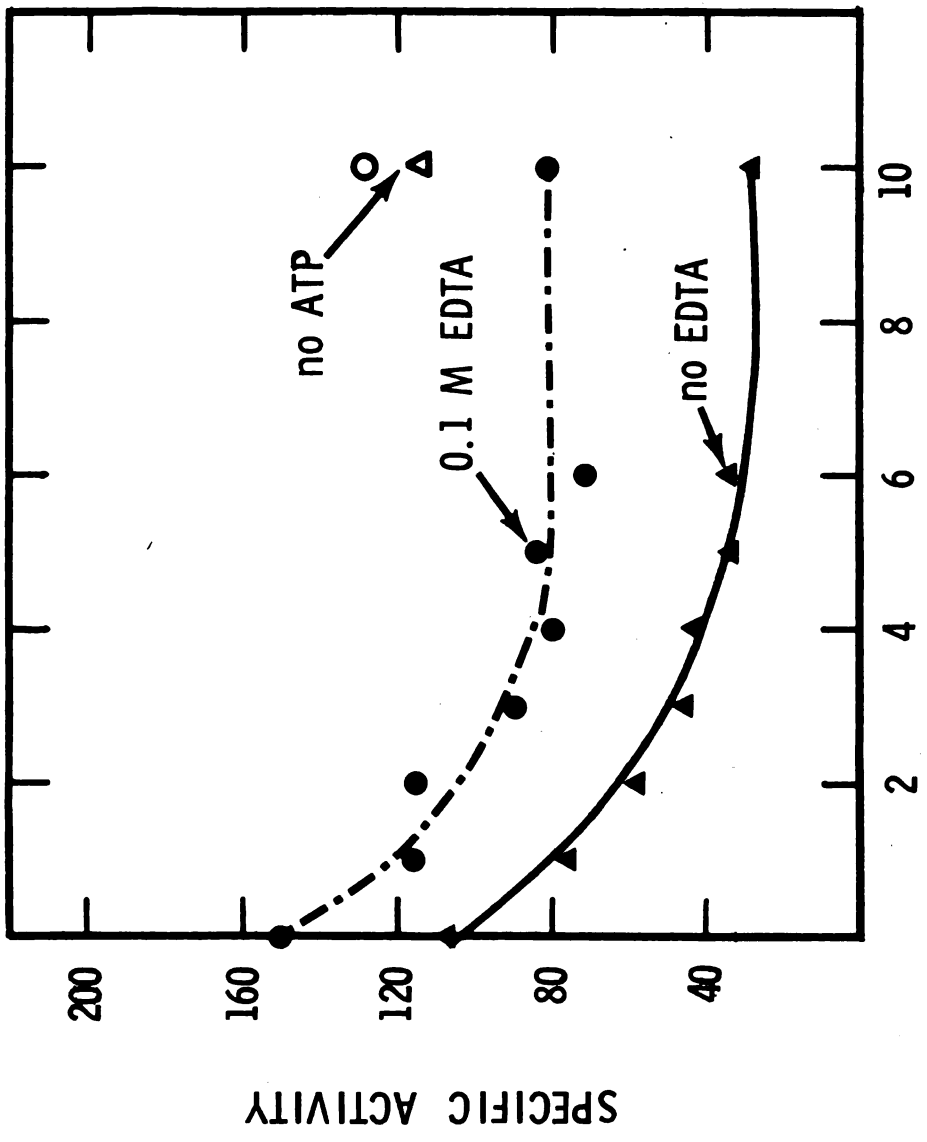
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)
no ATP
0.1 M EDTA
no EDTA

Figure 5

recovered at 0°, while from the gradient tube containing no EDTA and no ATP, no activity was recovered.

Quite similar values, 5.0 and 4.6 S respectively, were obtained for the sedimentation coefficients of GAPD dissociated by ATP at 0° in the presence and absence of EDTA. Thus EDTA did not protect against dissociation as it did against inactivation. In the absence of ATP, sedimentation coefficients of 7.8 and 8.2 S respectively were observed in the presence and absence of 0.1 M EDTA at 0°.

EDTA had no effect on the activity or structure of native GAPD at 25° in the absence of ATP.

Effect of Protein Concentration. With decreasing protein concentration, GAPD was inactivated more readily by ATP at 0° (Figure 6). The increase in inactivation was not directly proportional to the decrease in protein concentration. After 10 hr incubation under inactivating conditions, the 1.0 mg/ml sample lost 28 per cent of its activity; the 0.1 mg/ml sample (10 per cent as much protein) lost 35 per cent of its activity; and the 0.025 mg/ml sample (4 per cent as much protein) lost 81 per cent of its activity.

Not only inactivation, but also dissociation increased with decreasing protein concentration (Figure 7). At 1.0 mg/ml, the dissociation pattern showed a single peak with a sedimentation coefficient of $S_{20,w}$ = 6.7 S.

Figure 6. Effect of protein concentration on inactivation of pig liver GAPD by ATP at 0°. Dissociation samples and controls were prepared and treated as described in the legend for Figure 2, except the GAPD concentration was varied.

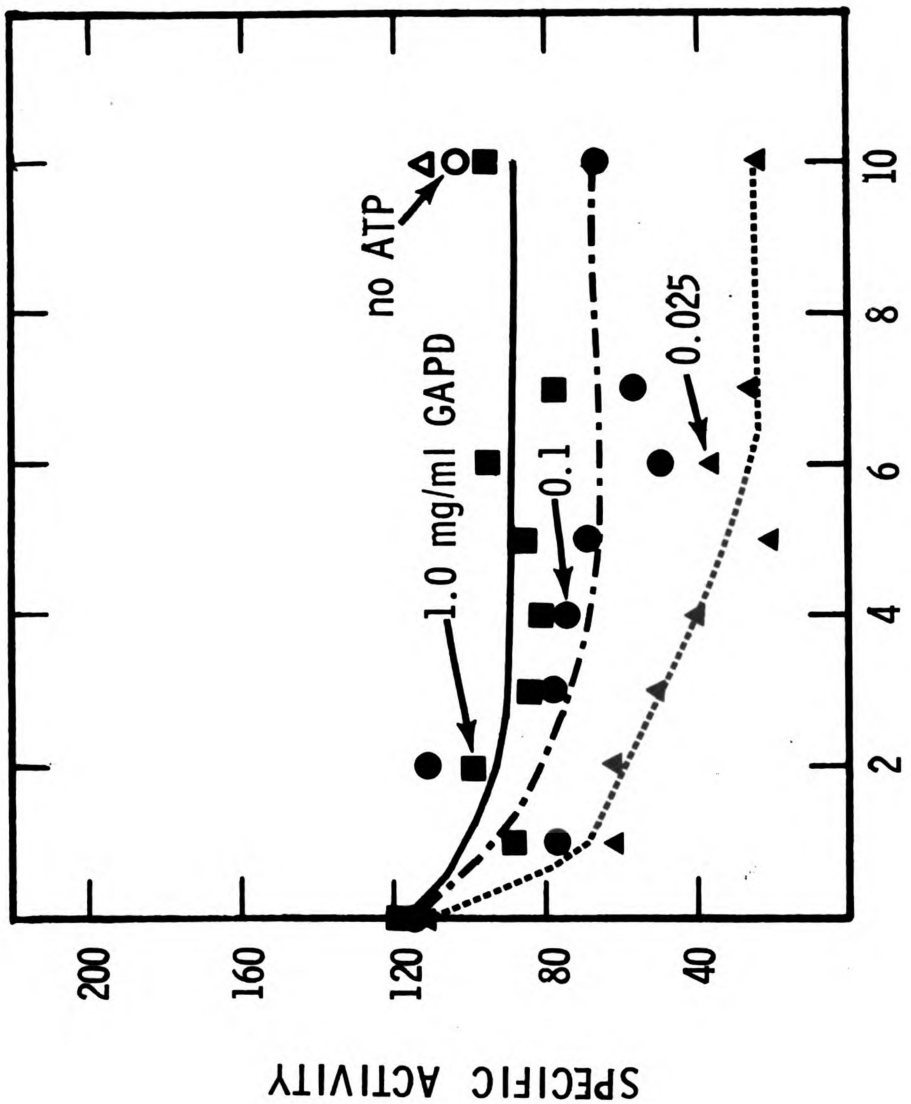
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)
no ATP
1.0 mg/ml GAPD
0.1
0.025

Figure 6

Figure 7. Sedimentation patterns for native (upper frame) and ATP-dissociated (lower frame) pig liver GAPD at various protein concentrations. These studies were carried out on the enzyme samples described in the legend for Figure 6. The samples were centrifuged in sucrose density gradients as described in the legend for Figure 3. The native enzyme samples (upper frame) are the controls described in the legend for Figure 6. The curves for the native enzyme at 1.0 and 0.1 mg/ml were identical, so only one curve is drawn for both sets. The $O.D._{650}$ values for 0.1 mg/ml and 0.025 mg/ml have been multiplied by 10 and 40 respectively to adjust them to the same scale as the 1.0 mg/ml values. This multiplication results in an over-correction because with each lower protein concentration, less reagent was used in protein determination and the resulting higher sucrose concentrations affected the $O.D._{650}$ readings.

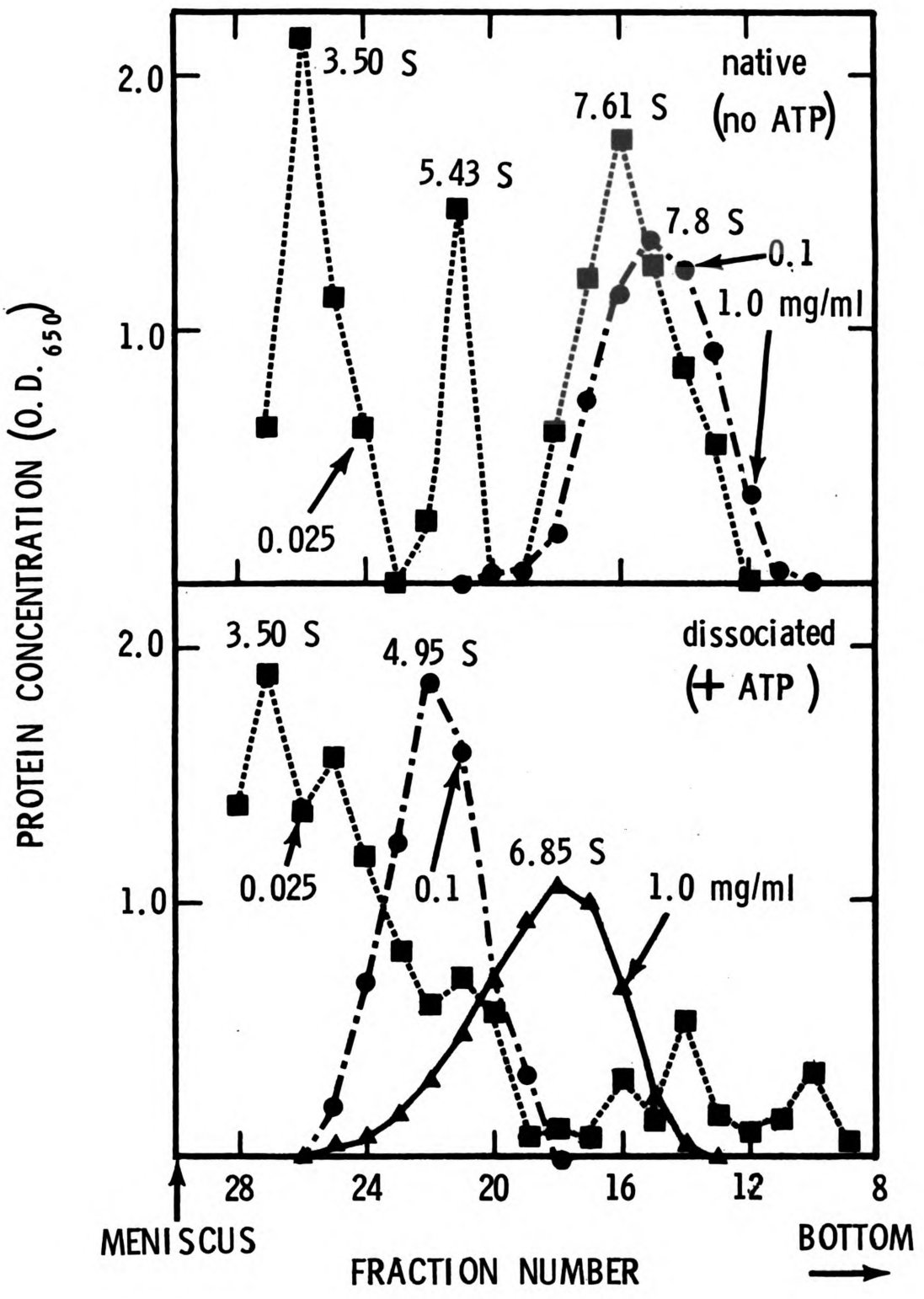
native
(no ATP)
3.50 S
7.61 S
5.43 S
7.8 S
0.1
1.0 mg/ml
0.025
dissociated
(+ ATP)
3.50 S
4.95 S
6.85 S
0.025
0.1
1.0 mg/ml
2.0
1.0
PROTEIN CONCENTRATION (O.D.650)
28
24
20
16
12
8
MENISCUS
BOTTOM
FRACTION NUMBER

Figure 7

At 0.1 mg/ml, a single 5.0 S peak was observed. Native GAPD at both concentrations had a sedimentation coefficient of $S_{20,w}$ = 7.8 S. But at 0.025 mg/ml, two peaks in a 1:6 ratio with sedimentation coefficients of 5.4 and 3.5 S respectively were observed. At this lowest concentration, the sedimentation pattern for native GAPD showed three peaks with a ratio of 2:1:1 and sedimentation coefficients of 7.6, 5.4 and 3.5 S respectively. Even after warming to 25°, no activity was observed in fractions from gradients containing ATP-dissociated enzyme whose original concentration was 0.025 mg/ml. But at 0°, 19 and 51 per cent respectively of the activity applied was recovered from gradients containing ATP and 0.1 or 1.0 mg/ml GAPD.

Effect of pH. The rate and extent of inactivation of GAPD by ATP at 0° was greater at pH values above and below pH 7.5 than they were at pH 7.5 (Figure 8). They were greatest at acid pH values. GAPD inactivated for 8 hr at pH 9.0 lost 45 per cent of its activity, GAPD inactivated at pH 7.5 lost 37 per cent of its activity, and that inactivated at pH 6.0 lost 88 per cent of its activity. The inactivation at pH 5.0 was even more extreme; all activity was lost within one hour. Without ATP, incubation of GAPD at 0° caused no loss of activity at pH 9.0 or 7.5, but there was significant loss at the acid pH values. At pH 6.0, GAPD incubated without ATP

Figure 8. Effect of pH on inactivation of pig liver GAPD by ATP at 0°. In these studies, the standard ATP inactivation system was used, except the buffer and pH were varied. The inactivation was carried out in 50 mM solutions of glycine, pH 9.0, imidazole, pH 7.5 or 6.0, and sodium acetate, pH 5.0. Otherwise, dissociation samples and controls were prepared and treated as described in the legend for Figure 2. It should be noted that these samples and controls (except the pH 5.0 control) were incubated for only 8 hr as compared to 10 hr in the other experiments.

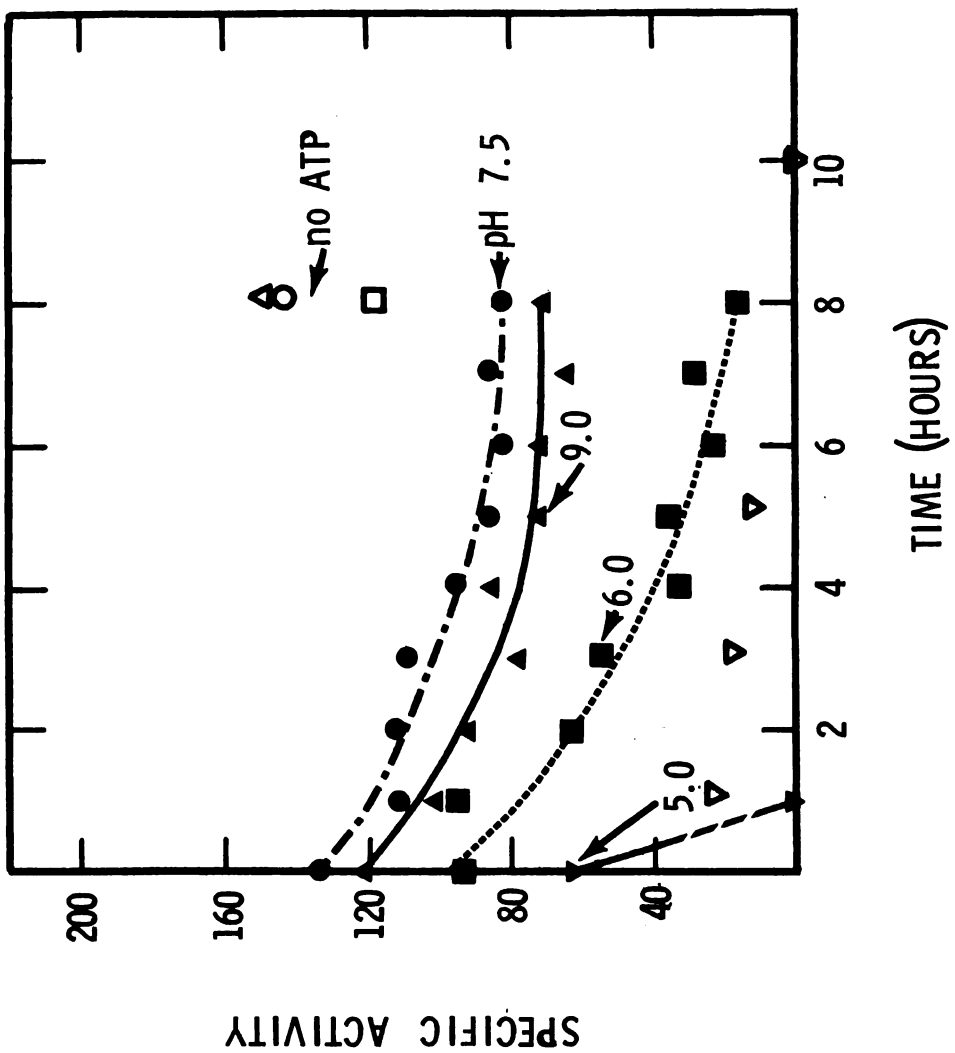
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)
no ATP
pH 7.5
9.0
6.0
5.0

Figure 8

lost 18 per cent of its activity after 8 hr; at pH 5.0 it lost all its activity after 10 hr. Thus the increased inactivation at acid pH values was caused by the acidic media as well as the presence of ATP.

The enzyme sample at pH 7.5 showed a 5.6 S peak (Figure 9), while that at pH 9.0 yielded at 5.8 S peak. There was little difference in degree of inactivation or dissociation in the samples exposed to various pH values in the range of pH 7.5 to 9.0. In contrast, GAPD at pH 6.0 dissociated to a 4.9 S peak, suggesting it was more dissociated than GAPD at pH 7.5. However, GAPD incubated without ATP at pH 6.0 and 0° was also partially dissociated; it had a sedimentation coefficient of 6.8 S. The sedimentation patterns for these dissociations are shown in Figure 10. Enzyme incubated at pH 5.0 and 0° with and without ATP continuously dissociated throughout the experiment; they were spread throughout the entire gradients.

From the gradients with ATP, the recovery of the activity at 0° was quite poor. From gradients at pH 5.0 or 6.0, no activity was recovered. From those at pH 7.5 and 9.0, 27 and 10 per cent respectively of the activity applied was recovered.

Effect of ATP Concentration. Increasing the ATP concentration from 0 to 15 mM in the incubation medium increased the inactivation of GAPD at 0° (Figure 11), but increasing

Figure 9. Effect of pH on dissociation of pig liver GAPD by ATP at 0°. These studies were carried out on the enzyme samples described in the legend for Figure 8. The samples were centrifuged in sucrose density gradients as described in the legend for Figure 3. The pH 5.0 sample and control were denatured and had no sedimentation peaks; they are not represented in the figure.

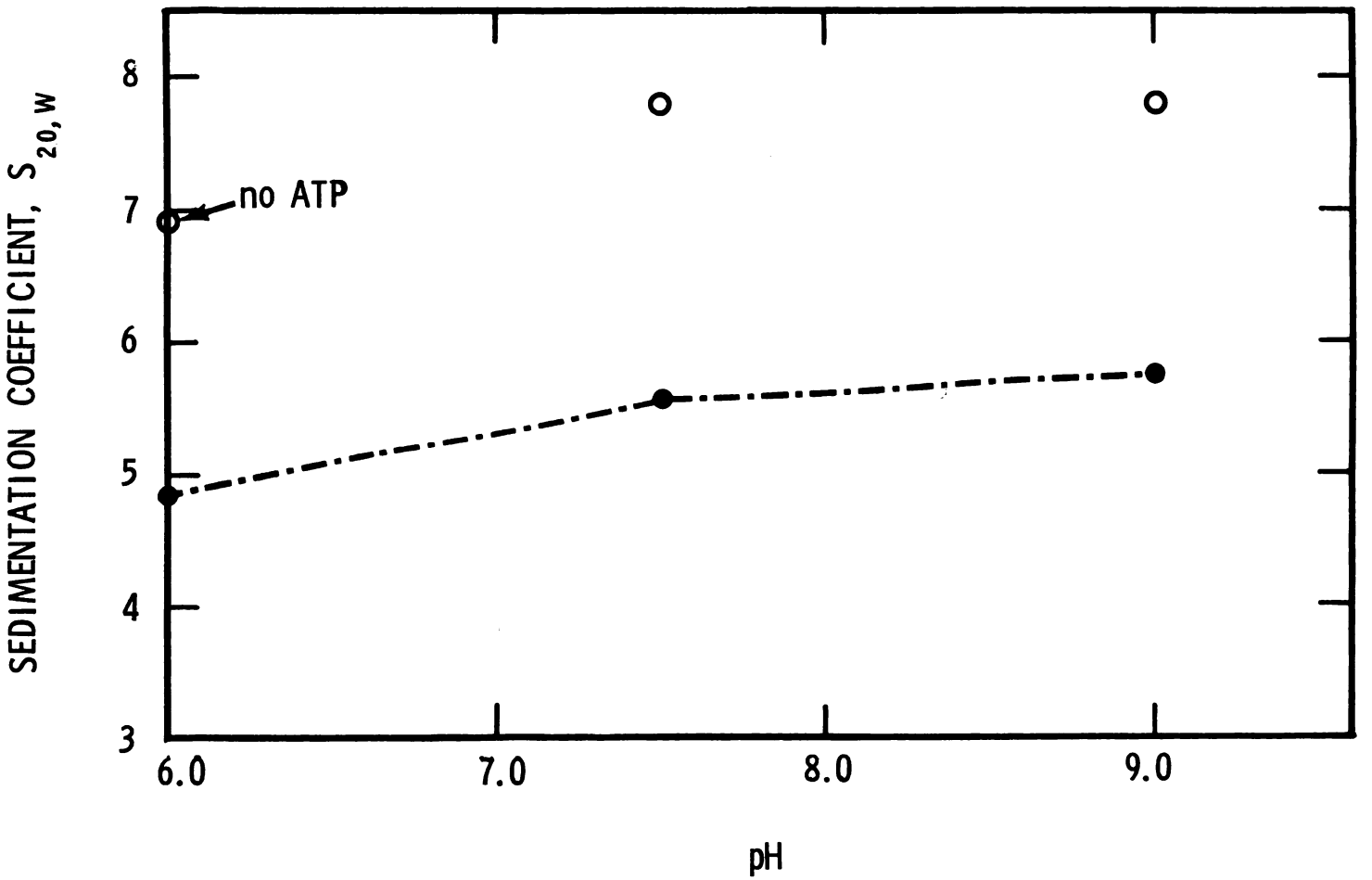

Figure 9

Figure 10. Sedimentation patterns for native (upper frame) and ATP-dissociated (lower frame) pig liver GAPD at different values of pH. These peaks are the sedimentation patterns obtained with inactivated and dissociated enzyme samples described in the legends for Figures 8 and 9. The native enzyme samples (upper frame) are the controls described in the legend for Figure 8. The curves for the native enzyme at pH 9.0 and 7.5 were identical, so only one curve is drawn for both sets. The OD_{650} values for pH 6.0 have been multiplied by 4 in an attempt to adjust them to the same scale as the values for pH 7.5 and 9.0. The protein determination does not work well at acid pH values.

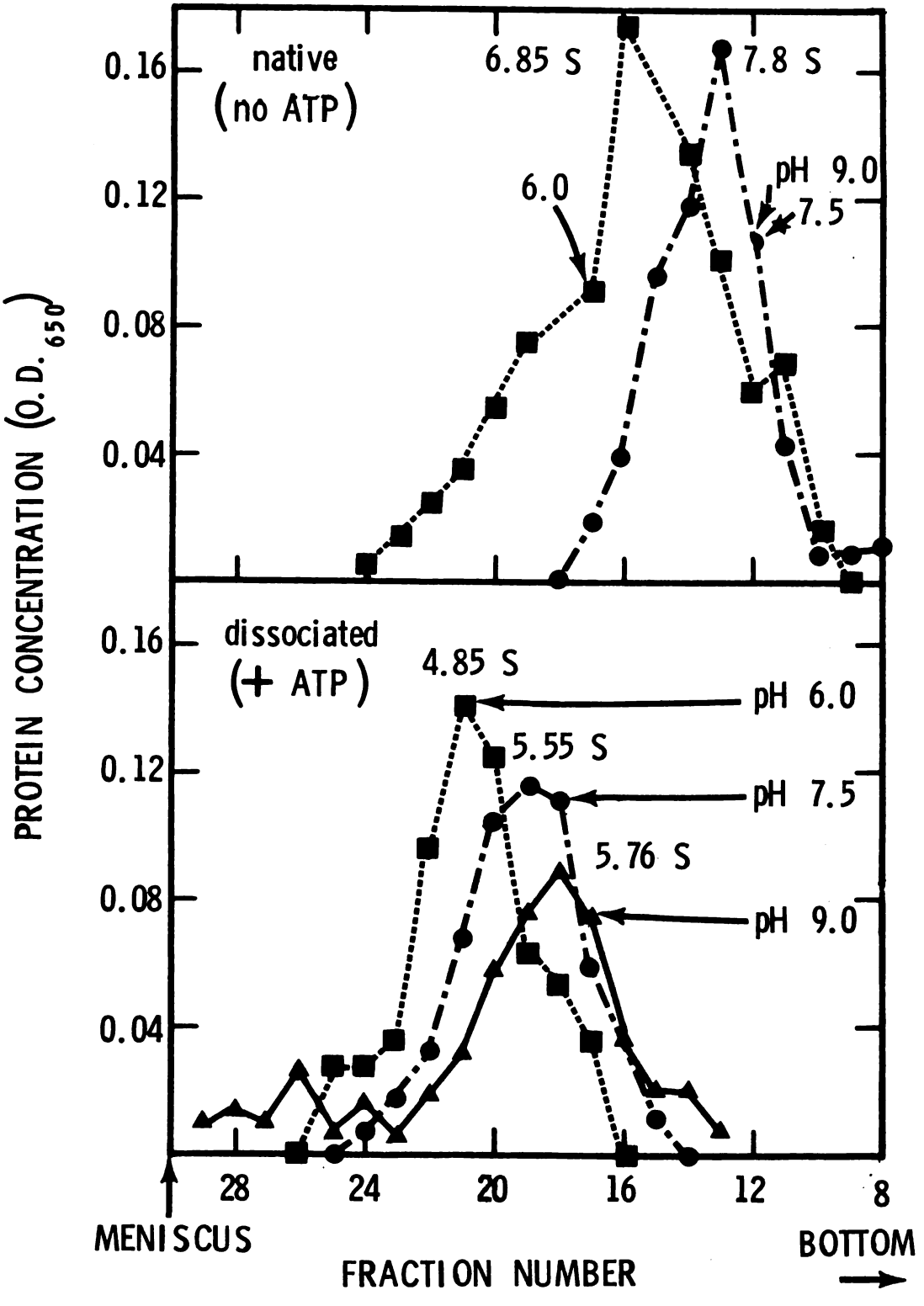
native
(no ATP)
6.85 S
7.8 S
6.0
pH 9.0
7.5
dissociated
(+ ATP)
4.85 S
pH 6.0
5.55 S
pH 7.5
5.76 S
pH 9.0
0.16
0.12
0.08
0.04
PROTEIN CONCENTRATION (O.D.650)
28
24
20
16
12
8
MENISCUS
BOTTOM
FRACTION NUMBER

Figure 10

Figure 11. Effect of ATP concentration on inactivation of pig liver GAPD by ATP at 0°. Aside from the variation in ATP concentration, dissociation samples and controls were prepared and treated as described in the legend for Figure 2.

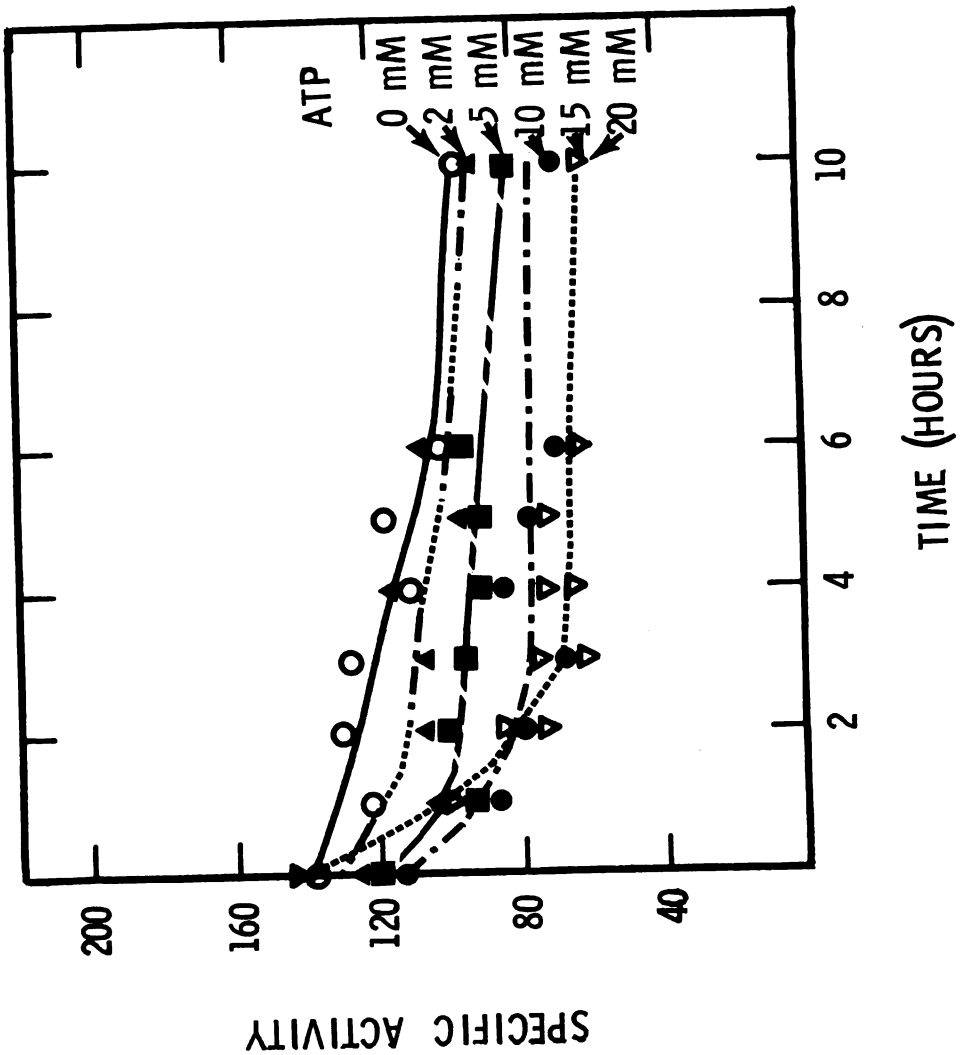
ATP
0 mM
2 mM
5 mM
10 mM
15 mM
20 mM
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)

Figure 11

the ATP concentration beyond 15 mM did not produce more inactivation. After 10 hr, the per cent activities lost from enzyme in solutions containing various ATP concentrations were as follows (activity loss given in parenthesis): 0 mM (30%), 2 mM (34%), 5 mM (42%), 10 mM (46%), 15 or 20 mM (56%).

The dissociation of GAPD also increased when the ATP concentration of the incubation medium was raised from 0 to 15 mM (Figure 12). Similarly, ATP concentrations above 15 mM caused no further dissociation. The sedimentation coefficients of GAPD dissociated at various ATP concentrations were as follows (sedimentation coefficients given in parenthesis): 0 mM (7.8 S), 2 mM (6.0 S), 5 mM (5.6 S), 10 mM (5.0 S) and 15 or 20 mM (4.7 S). The rate of decrease in sedimentation coefficient with increase in ATP concentration was more pronounced than the rate of loss of activity.

The recovery of activity from the gradients at 0° decreased steadily with increasing ATP concentration. The per cent recoveries at 0° of the activity applied to the gradients were as follows (per cent recovery given in parenthesis): 0 mM (42%), 2 mM (15%), 5 mM (12%), 10 mM (11%), 15 mM (10%) and 20 mM (5%).

Effect of Mercaptoethanol. Mercaptoethanol partially protected against inactivation of GAPD in solutions containing ATP at 0° (Figure 13). Up to 40 mM, protection

Figure 12. Effect of ATP concentration on dissociation of pig liver GAPD by ATP at 0°. These studies were carried out on the enzyme samples described in the legend for Figure 11. The samples were centrifuged in sucrose density gradients as described in the legend for Figure 3.

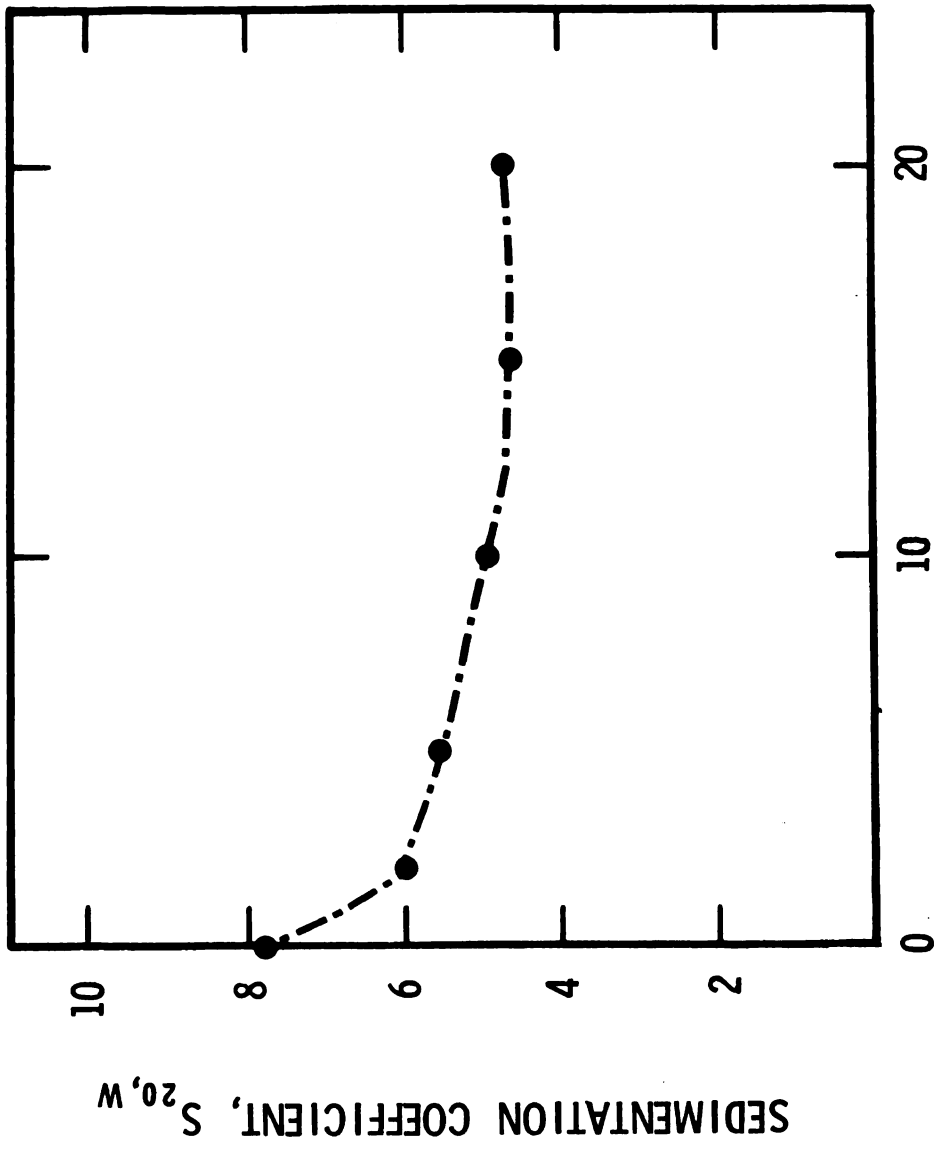
SEDIMENTATION COEFFICIENT, $S_{20,w}$
10
8
6
4
2
0
10
20
CONCENTRATION of ATP (mM)

Figure 12

Figure 13. Effect of mercaptoethanol concentration on inactivation of pig liver GAPD by ATP at 0°. Dissociation samples and controls were prepared and treated as described in the legend for Figure 2, except the mercaptoethanol concentration was varied. The sedimentation coefficients measured for these enzyme solutions (obtained by sucrose density gradient centrifugation as described in the legend for Figure 3) are presented in the text. The curves for the dissociated enzyme at 40 mM and 80 mM mercaptoethanol were identical, so only one curve is drawn for both sets.

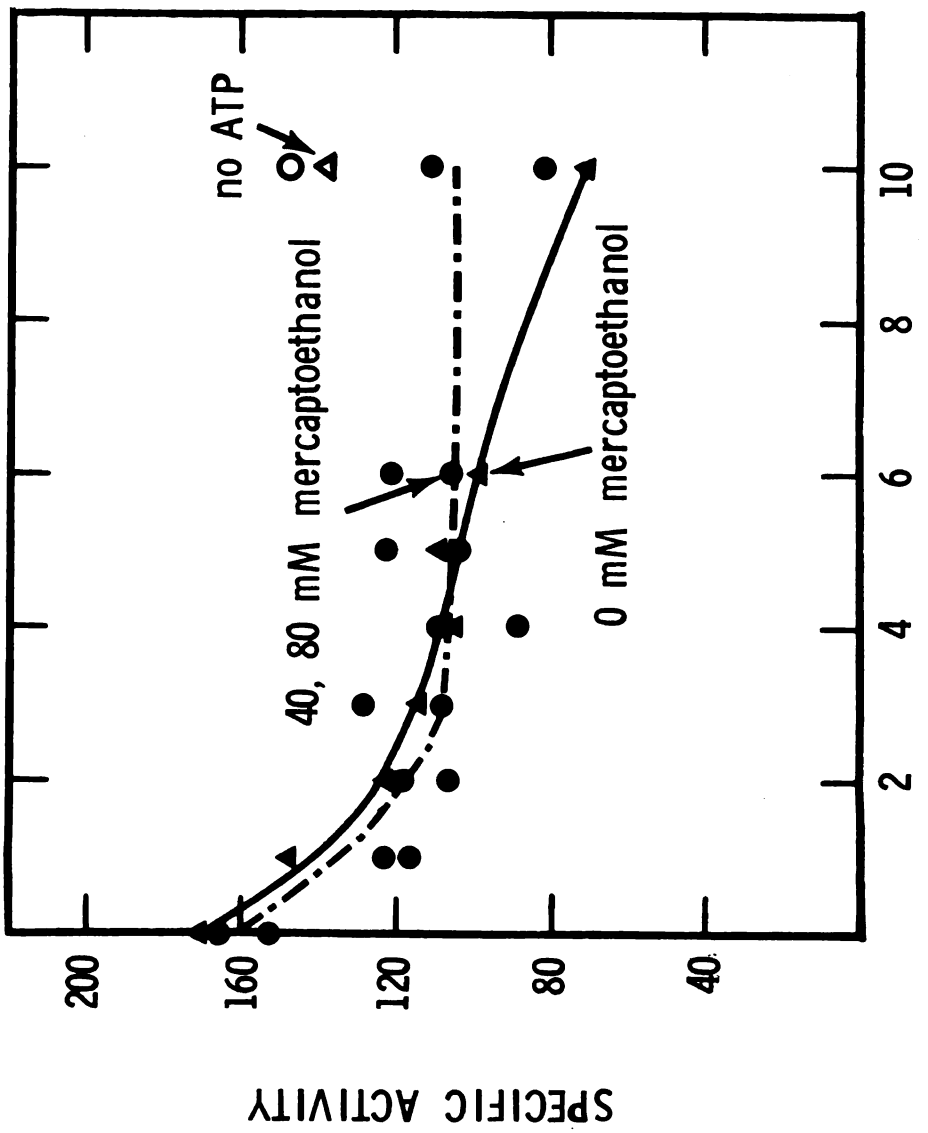
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)
no ATP
40, 80 mM mercaptoethanol
0 mM mercaptoethanol

Figure 13

increased with increasing mercaptoethanol concentration, but concentrations greater than 40 mM gave no better protection than 40 mM. In 0 mM mercaptoethanol GAPD had a sedimentation coefficient of 4.6 S, while in 40 mM and 80 mM mercaptoethanol it had sedimentation coefficients of 5.3 and 5.2 S respectively. The recovery of activity from the gradients at 0° was 5 per cent of the activity applied in 0 mM mercaptoethanol, 17 per cent of the activity applied in 40 mM, and 23 per cent of the activity applied in 80 mM.

Effect of Temperature. Increasing the temperature of incubation from the normal 0° to 25° caused a marked decrease in inactivation by ATP (Figure 14). After 10 hr, GAPD incubated with ATP at 25° had lost no activity, but GAPD incubated at 12° and 0° had lost 43 and 50 per cent of its activity respectively. The rate of loss of activity at 12° was more linear with time than the rate of loss at 0°.

The sedimentation coefficient decreased linearly with the decrease in temperature (Figure 15). The GAPD at 0° had a sedimentation coefficient of 5.2 S, that at 12° had a sedimentation coefficient of 5.5 S and the enzyme at 25° had a sedimentation coefficient of 5.8 S (Figure 16).

It is perhaps surprising that GAPD incubated with ATP at 25° showed no loss in activity, but showed a substantial decrease in sedimentation coefficient

Figure 14. Effect of temperature on inactivation of pig liver GAPD by ATP. In these studies, the standard ATP inactivation system was used except the temperature was varied. Otherwise, dissociation samples and controls were prepared and treated as described in the legend for Figure 2.

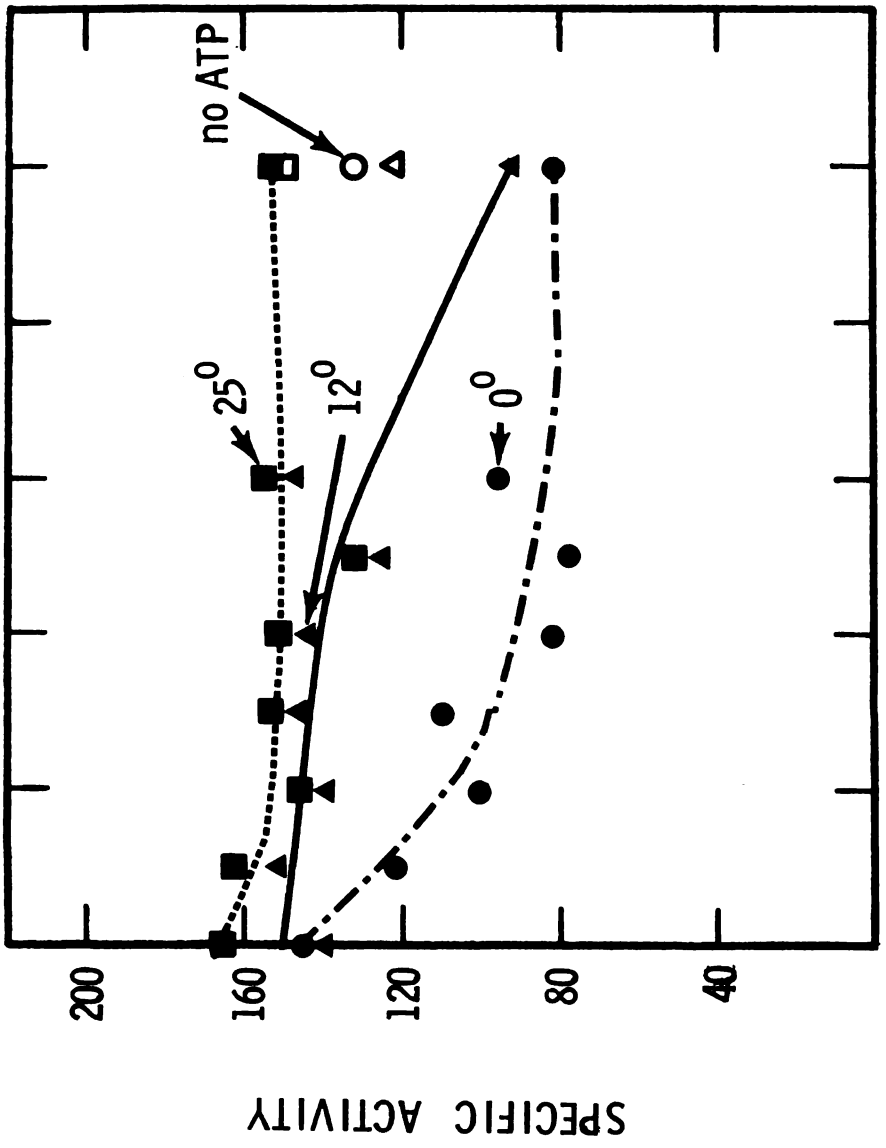
no ATP
25°
12°
0°
SPECIFIC ACTIVITY
200
160
120
80
40
2
4
6
8
10
TIME (HOURS)

Figure 14

Figure 15. Effect of temperature on dissociation of pig liver GAPD by ATP. These studies were carried out on the enzyme samples described in the legend for Figure 14. The samples were centrifuged in sucrose density gradients as described in the legend for Figure 3, except that centrifugation of each set of samples was carried out at the same temperature as that used for inactivation. The 25° gradients were centrifuged at 45,000 rpm for 7.75 hr; the 12° gradients were centrifuged at 45,000 rpm for 10.5 hr; and the 0° gradients were centrifuged at 45,000 rpm for 13.5 hr. All the controls had sedimentation coefficients of $S_{20,w}$ = 7.8 S (not plotted). The sedimentation coefficient at 25° may be low (see text).

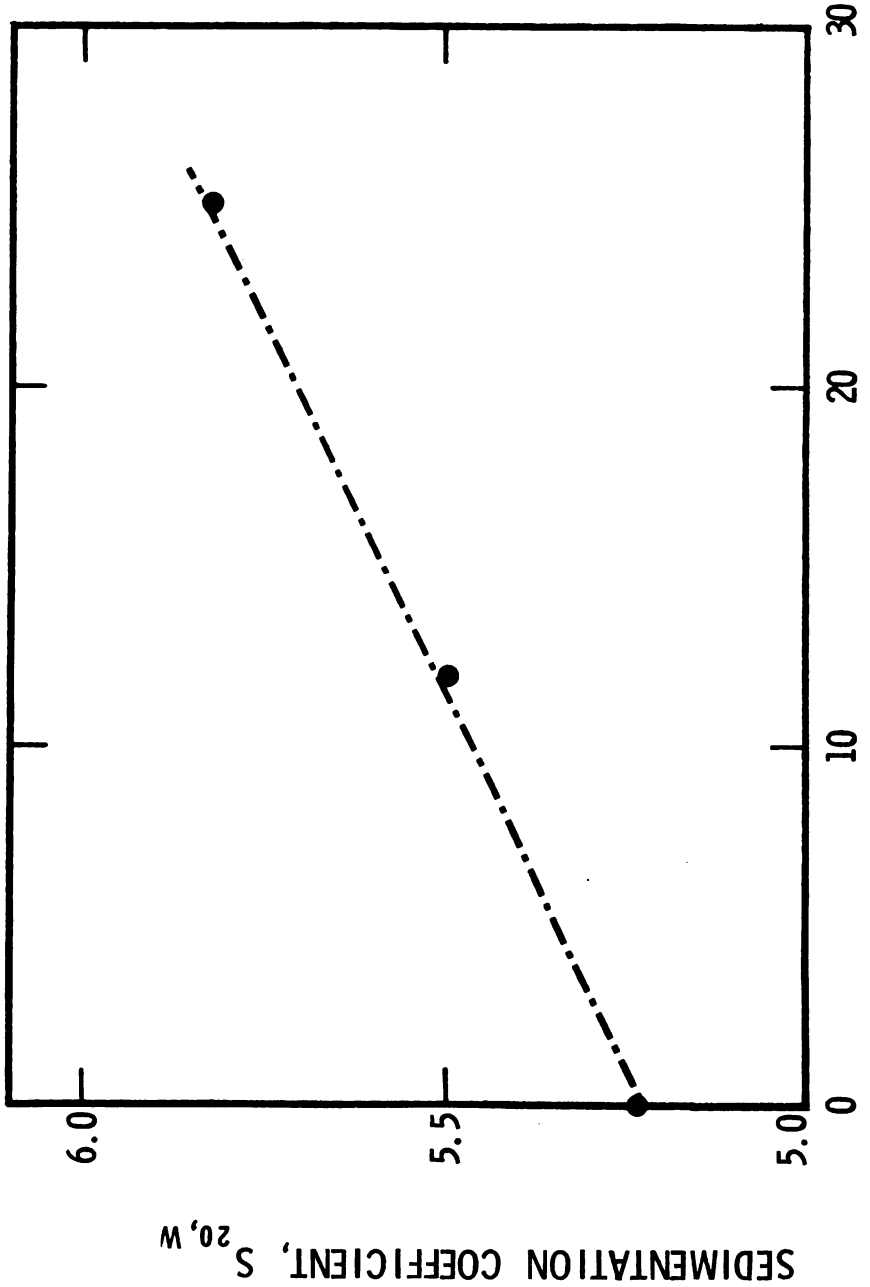
SEDIMENTATION COEFFICIENT, $S_{20,w}$
6.0
5.5
5.0
0
10
20
30
TEMPERATURE

Figure 15

Figure 16. Sedimentation patterns for native and ATP-dissociated pig liver GAPD at different temperatures. These peaks are the sedimentation pattern obtained with the inactivated and dissociated enzyme samples described in the legends for Figures 14 and 15. The native sample is the control described in the legend for Figure 14 (all the controls gave the same curve).

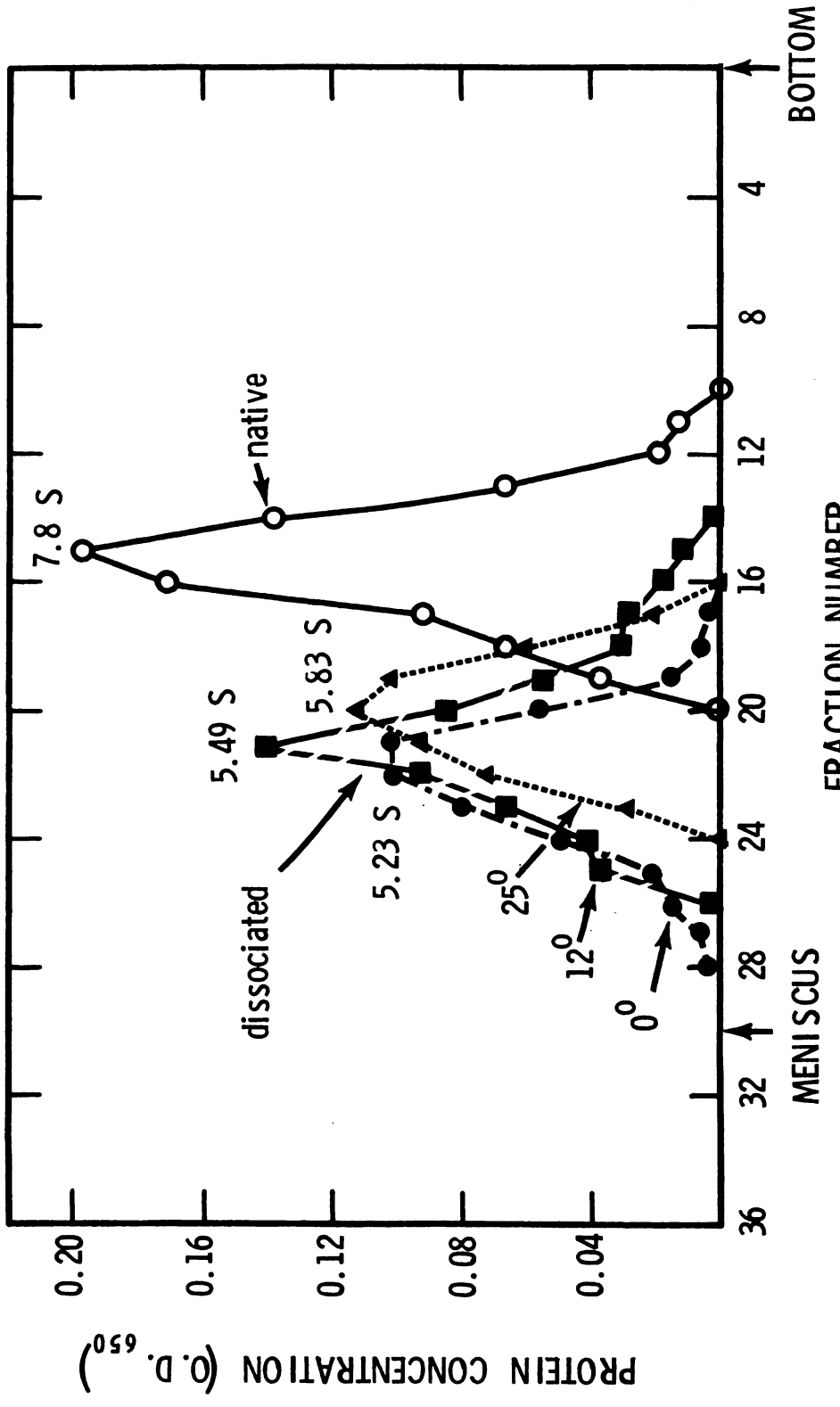
PROTEIN CONCENTRATION (O.D.650)
0.20
0.16
0.12
0.08
0.04
36
32
28
24
20
16
12
8
4
MENISCUS
FRACTION NUMBER
BOTTOM
7.8 S
native
5.83 S
5.49 S
5.23 S
dissociated
25°
12°
0°

Figure 16

(compared to native GAPD). Although these samples were not assayed in the presence of ATP, there was no evidence for reactivation during the assay. Hence, reassociation and reactivation in the assay seems ruled out as an explanation for the results. A more likely explanation is that the GAPD dissociated during centrifugation. This explanation is supported by the facts that GAPD incubated with ATP at 25° and GAPD incubated without ATP at 25° had identical activities (within experimental error) before sedimentation, but enzyme with ATP had 80 per cent less activity than enzyme without ATP after sedimentation. Similar results were observed with fully reactivated GAPD centrifuged in the presence of ATP (see next section).

REACTIVATION AND REASSOCIATION OF GLYCERALDEHYDE 3-P DEHYDROGENASE. The conditions required for the reactivation and reassociation of yeast (Stancel and Deal, 1969) and rabbit muscle GAPDs (Constantinides and Deal, 1969) after inactivation and dissociation by ATP at 0° are well defined. These conditions, however, are not adequate to reactivate and reassociate similarly treated pig liver GAPD (Dagher and Deal, in preparation). The idea that EDTA might be the missing required compound was suggested by the discovery that EDTA increased the activity of pig liver GAPD under inactivating and dissociating conditions (see section "Effect of EDTA").

Consequently, a short study was undertaken to characterize the reactivation and reassociation of GAPD in the presence of EDTA.

The importance of EDTA for the reactivation of GAPD can be seen in the first two lines of Table 2; the per cent activity regained was two fold greater in the presence of EDTA than in its absence. To affect the reactivation, the EDTA had to be present throughout both the inactivation and reactivation; it could not be added for just the reactivation by warming to 25° (first three lines, Table 2).

Since regain of activity was not 100 per cent even in the presence of EDTA, a systematic study to see whether the addition of other compounds to the EDTA-containing reactivation solution could produce complete recovery of activity was undertaken (lines 4, 5 and 6, Table 2). Adding NAD improved the reactivation; adding sucrose decreased it. Together they cancelled each other's effects and gave no change in reactivation. The last line of Table 2 illustrates the importance of reducing agent to reactivation; reactivation in the absence of mercaptoethanol was only slightly better than in the absence of EDTA.

A study of the effect of incubation time at 0° on reactivation is shown in Table 3. In most cases, little difference was seen between inactivating for 12

Table 2. Reactivation of Inactivated GAPD in the Presence of Different Compounds

Number of Samples	EDTA[a]	Mercapto-ethanol[a]	Compounds Added to Aid Reactivation[b]	Per Cent Original Activity Regained[c]
three	-	+	no addition	38.6 - 52.7
two	+	+	no addition	80.5 - 88.6
one	-	+	0.1 M EDTA	46.3
two	+	+	5 mM NAD	90.9 - 100
one	+	+	10% sucrose	73.4
one	+	+	5 mM NAD, 10% sucrose	85.7
one	+	-	5 mM NAD, 10% sucrose	53.5

[a]Standard inactivation medium contained 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol and 10 mM ATP.

[b]Compounds were added to the 0° inactivated GAPD just before warming to 25° for reactivation.

[c]Samples were inactivated by incubating at 0° with ATP for 10 to 12 hr and reactivated by warming to 25° for 5 min. The samples did not completely inactivate.

Table 3. The Effect of Incubation Time at 0° on Reactivation[a]

Compounds Added to or Omitted from the Standard Inactivation Solution[b]	Per Cent Activity Regained After 12 Hr Inactivation[c]	Per Cent Activity Regained After 24 Hr Inactivation[c]	Per Cent Activity Regained After 36 Hr Inactivation[c]
standard solution	88.6	90.0	76.4
+ 10% sucrose	73.4	74.4	62.9
+ 5 mM NAD	100	84.3	66.1
+ 10% sucrose, 5 mM NAD	85.7	74.4	64.0
+ 10% sucrose, 5 mM NAD, - mercaptoethanol	53.5	51.7	49.9

[a]Enzyme was reactivated by warming to 25° for 5 min.

[b]Standard inactivation solution contained 50 mM imidazole, 0.1 M EDTA pH 7.5, 80 mM mercaptoethanol and 10 mM ATP. Added compounds were introduced just before beginning reactivation. Omitted compounds were left out for both inactivation and reactivation.

[c]Enzyme was inactivated by incubating at 0° for the times indicated. The samples did not completely inactivate.

and 24 hr, but a significant loss in per cent activity regained occurred after inactivating for 36 hr. An exception to this trend was reactivation in the presence of NAD; it showed a steady decline in per cent activity regained upon additional incubation at 0° for several hours.

The kinetics of reactivation in the presence of EDTA after inactivating for 12 hr are shown in Figure 17. The kinetics after inactivating for 24 and 36 hr were very similar. Maximal activity was reached in 3.5 to 5 min, a remarkably short time compared to the 12 hr required for inactivation (Figure 2).

The sedimentation behavior of reactivated GAPD was examined in sucrose density gradients to see whether the reactivated enzyme had also reassociated. Fully reactivated enzyme centrifuged in gradients without ATP showed a pattern like that in Figure 18. The sedimentation coefficients of native and reassociated GAPD were identical, within experimental error, indicating that the enzyme was completely reassociated as well as reactivated. However, fully reactivated GAPD centrifuged in gradients containing ATP showed a pattern like that in Figure 19. Native GAPD had a sedimentation coefficient of 7.8 S, but reactivated GAPD had a sedimentation coefficient of 5.4 S.

Figure 17. Kinetics of reactivation of pig liver GAPD inactivated by ATP at 0°. Dissociation samples were prepared and treated as described in the legend for Figure 2, except one sample had no mercaptoethanol. The incubation time was 12 hr. After inactivation by incubation at 0°, the samples were adjusted to the appropriate NAD and sucrose concentrations using 0.2 M NAD and 75% sucrose and then placed at 25° to reactivate. The activity values were corrected for the difference in volume caused by adding NAD or sucrose. The kinetics of reactivation after 24 or 36 hr inactivation were similar.

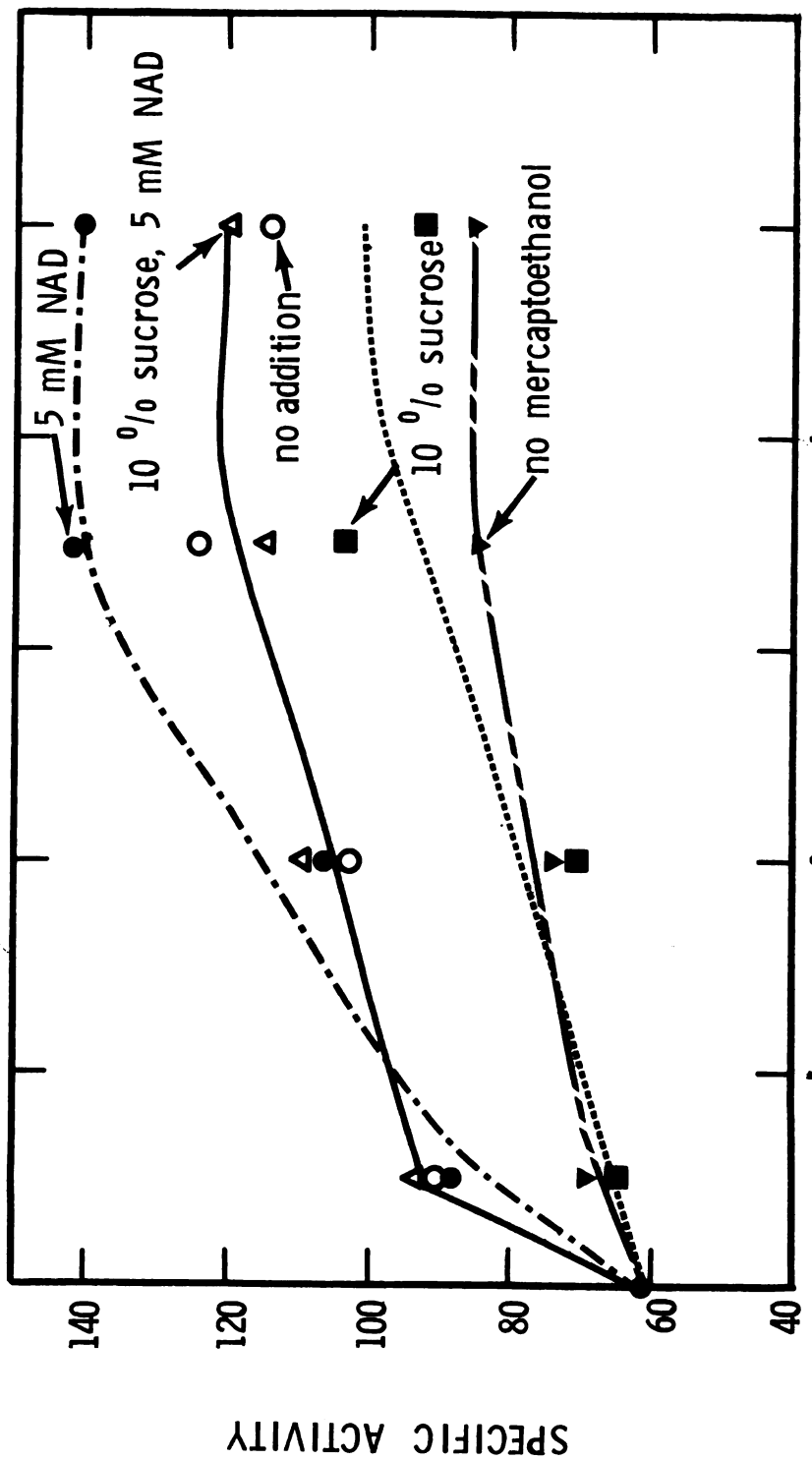
SPECIFIC ACTIVITY
140
120
100
80
60
40
1
2
3
4
5
TIME (MINUTES) at 25°
5 mM NAD
10 % sucrose, 5 mM NAD
no addition
10 % sucrose
no mercaptoethanol

Figure 17

Figure 18. Sedimentation pattern of reassociated pig liver GAPD centrifuged without ATP. A dissociation sample and its control were prepared and treated as described in the legend for Figure 2; the incubation time was 12 hr. After incubation, the dissociation sample was adjusted to 5 mM NAD using 0.2 M NAD. After warming to 25° for 5 min, the sample and control were centrifuged in sucrose density gradients as described in the legend for Figure 3, except the centrifugation was at 45,000 rpm for 7.75 hr at 25° and neither gradient contained ATP. The samples had identical activity after centrifugation (not plotted).

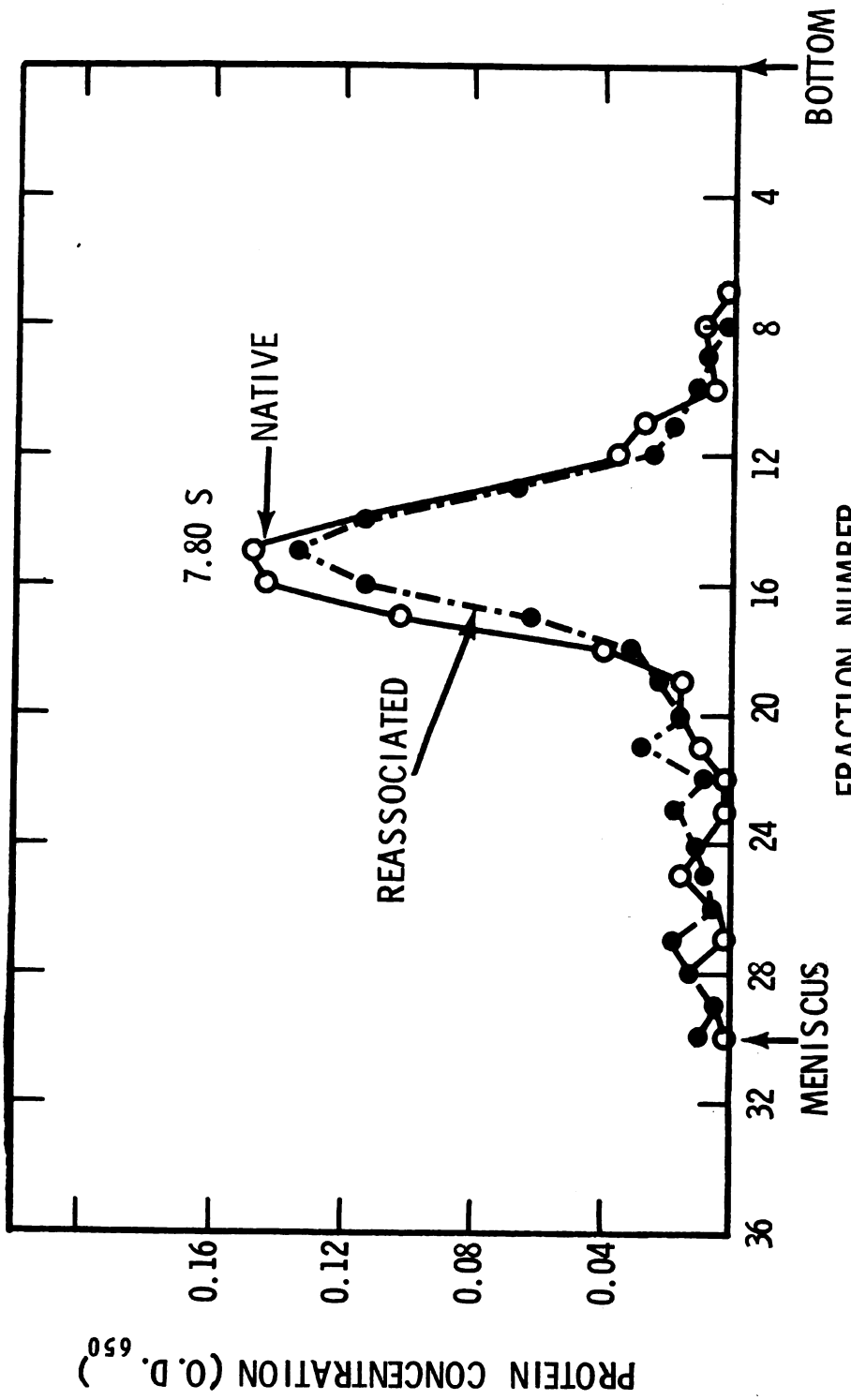
PROTEIN CONCENTRATION (O.D.650)
0.16
0.12
0.08
0.04
36
32
28
24
20
16
12
8
4
MENISCUS
FRACTION NUMBER
BOTTOM
7.80 S
NATIVE
REASSOCIATED

Figure 18

Figure 19. Sedimentation pattern of reassociated pig liver GAPD centrifuged with ATP. A dissociation sample and its control were prepared and treated as described in the legend for Figure 2; the incubation time was 12 hr. After incubation, the dissociation sample and control were warmed to 25° for 5 min; they had similar activities. The enzyme solutions were centrifuged in sucrose density gradients as described in the legend for Figure 3, except the centrifugation was at 45,000 rpm for 7.75 hr at 25°. The activity plotted is that measured at 25°. A discussion of the difference in sedimentation patterns between Figures 18 and 19 is presented in the text.

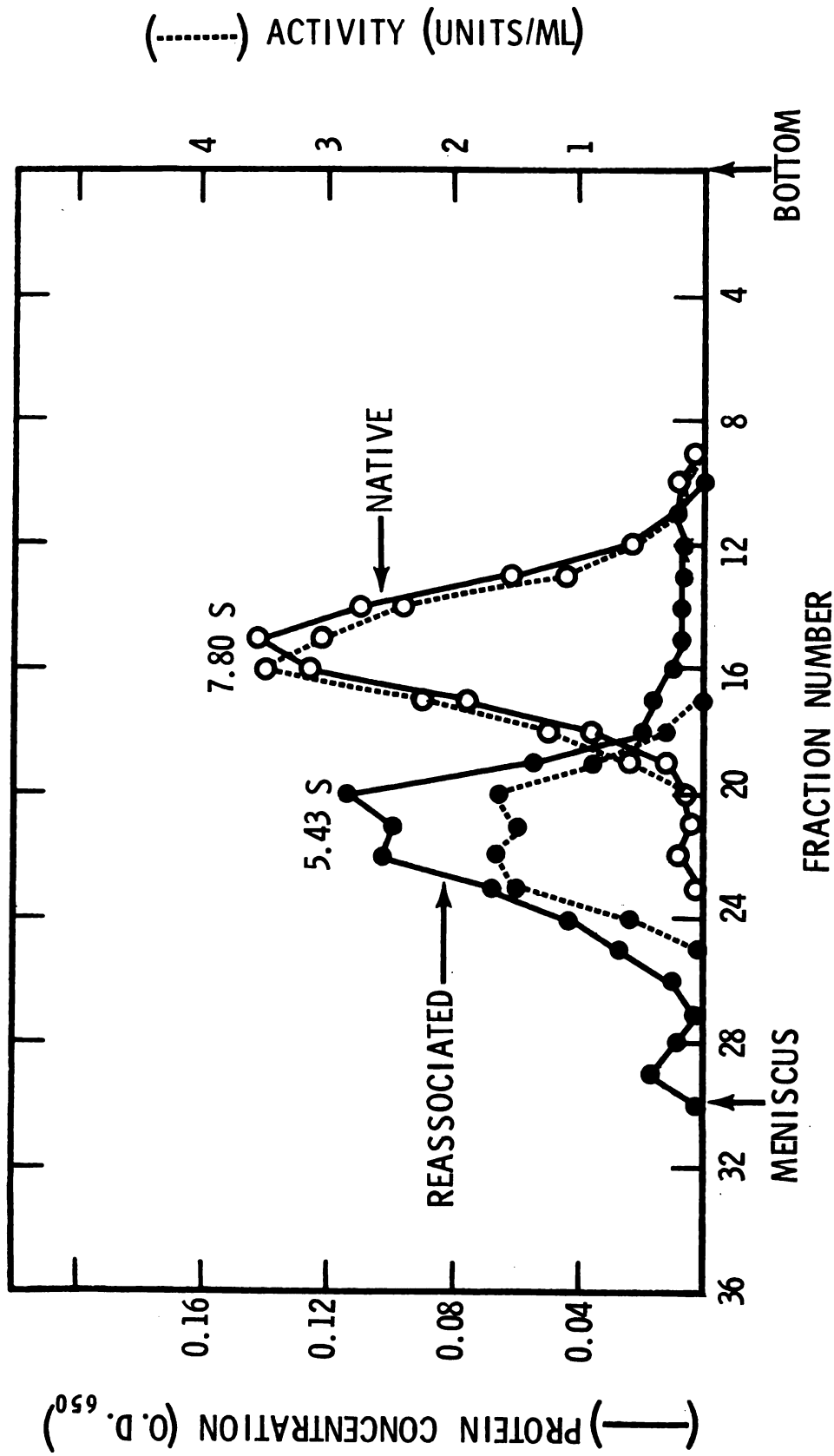
(.....) ACTIVITY (UNITS/ML)
4
3
2
1
BOTTOM
NATIVE
7.80 S
5.43 S
REASSOCIATED
MENISCUS
FRACTION NUMBER
4
8
12
16
20
24
28
32
36
0.16
0.12
0.08
0.04
(——) PROTEIN CONCENTRATION (O.D.650)

Figure 19

One explanation for the different sedimentation pattern in the presence of ATP is that ATP prevents reassociation, but not reactivation. This explanation seems unlikely because reactivated GAPD and native GAPD had identical activities (within experimental error) before sedimentation, but reactivated enzyme had 50 per cent less activity than native enzyme after sedimentation. If ATP prevents reassociation but not reactivation, this activity loss would not be expected. A more likely explanation of the different sedimentation pattern is that the reactivated enzyme was inactivated and dissociated in the gradient containing ATP. This explanation also agrees with similar results obtained for dissociation of GAPD at 25° with ATP (see section "Effect of Temperature"). No experiments with native enzyme in ATP containing gradients were performed, so it is not known whether native enzyme will also inactivate and dissociate under these conditions.

AMINO ACID COMPOSITION. To provide further chemical characterization of pig liver GAPD, the amino acid composition was determined (Table 4). The values listed represent a summary of data obtained from 18 hr and 56 hr hydrolyses. Threonine, serine, methionine and tyrosine were gradually degraded during hydrolysis; the values obtained at 18 hr and 56 hr were extrapolated back to 0 time to yield the ratios used in calculating the

Table 4. Amino Acid Composition

Amino Acid	Residues per Monomer	
	Pig Liver GAPD	Pig Muscle GAPD[a]
aspartic acid[b]	37.3	38
threonine	20.1	22
serine	18.9	19
glutamic acid[b]	20.9	18
glycine	37.0	32
alanine	32.1	32
valine	32.3	34
methionine	12.0	9
isoleucine	19.4	21
leucine	20	18
tyrosine	10.5	9
phenylalanine	13.5	14
ammonia	57.2[c]	18
lysine	23.5	26
histidine	9.0	11
arginine	10.2	10
proline	21.1	12
cysteine	3.8	4
tryptophan	4.7	3

[a]Data of Harris and Perham (1968).

[b]No information is available concerning the number of amidated residues, although typically they are about 20-30% of the total.

[c]This value may be high because no attempt was made to remove ammonia from the HCl used in hydrolysis.

values given in the table. Valine, isoleucine and lysine were not completely released by the 18 hr hydrolysis; the concentrations at 56 hr were used directly to calculate the values listed. All other amino acid values were an average of the sample hydrolyzed for 18 hr and that hydrolyzed for 56 hr. Residues were calculated from nanomoles by assigning the arbitrary value of 20 to leucine. (Leucine was chosen because its value remained constant for both hydrolyses. Twenty was chosen because the number of the various amino acid residues, calculated from 20 leucines, agreed favorably with the number expected, assuming all GAPDs are similar.)

The total number of residues calculated per monomer was 346. Using the molecular weights of the individual amino acids, a monomeric molecular weight of 38,070 daltons and a tetrameric molecular weight of 152,300 daltons are calculated. These results are in reasonable agreement with the values of 38,000 (monomer) and 148,000 (tetramer) obtained by Dr. Dagher using SDS polyacrylamide gel electrophoresis and high-speed sedimentation equilibrium respectively (Dagher and Deal, in preparation).

A partial specific volume of 0.7376 cc/g was calculated from the amino acid composition (method of Cohn and Edsall [1943] as described by Kayne [1966]). This value is very similar to the partial specific volume

of 0.737 cc/g at 20° measured for pig muscle GAPD (Elodi, 1958). The partial specific volume of most proteins is between 0.70 and 0.75 cc/g (Schachman, 1957).

The amino acid composition of pig muscle GAPD is also presented in Table 4 for comparison with that of pig liver GAPD. The two compositions are quite similar, except that pig liver GAPD has more proline. Pig muscle GAPD has only 332 residues per monomer (13 less than pig liver GAPD) and its monomer has a molecular weight of 36,000 (compared to 38,070 for pig liver).

SULFHYDRYL REACTIVITY OF NATIVE GLYCERALDEHYDE 3-P DEHYDROGENASE. Because sulfhydryl groups frequently play important roles in maintaining the structure of proteins and catalyzing enzymic reactions, determination of the number of sulfhydryl groups, their reactivity and their location within a given protein molecule are of interest. The number, reactivity and location of these groups are often determined by analyzing their reactivity toward sulfhydryl reagents since this reactivity depends heavily on the number of groups, how reactive they are and how accessible they are to the solvent.

The titration of available sulfhydryl groups with DTNB is shown in Figure 20. The first four groups reacted almost instantly (in less than 5 sec). The second four sulfhydryl groups reacted at a moderate

Figure 20. Sulfhydryl reactivity of native pig liver GAPD. GAPD was titrated with DTNB as described in Materials and Methods. The reaction mixture contained 240 µg GAPD, 200 µg EDTA and 40 µg DTNB (65 fold excess compared to GAPD) in 0.08 M sodium phosphate buffer, pH 8.0. The number of sulfhydryl residues titrated per tetramer was calculated with the molar extinction coefficient of 13,600 at 412 nm. The titration of the first four sulfhydryl groups occurred in less than 5 sec. The precipitation of the reacted enzyme was confirmed by measuring turbidity at 650 nm. The titration curve after precipitation began is more a measure of light scattering than of sulfhydryl groups reacted.

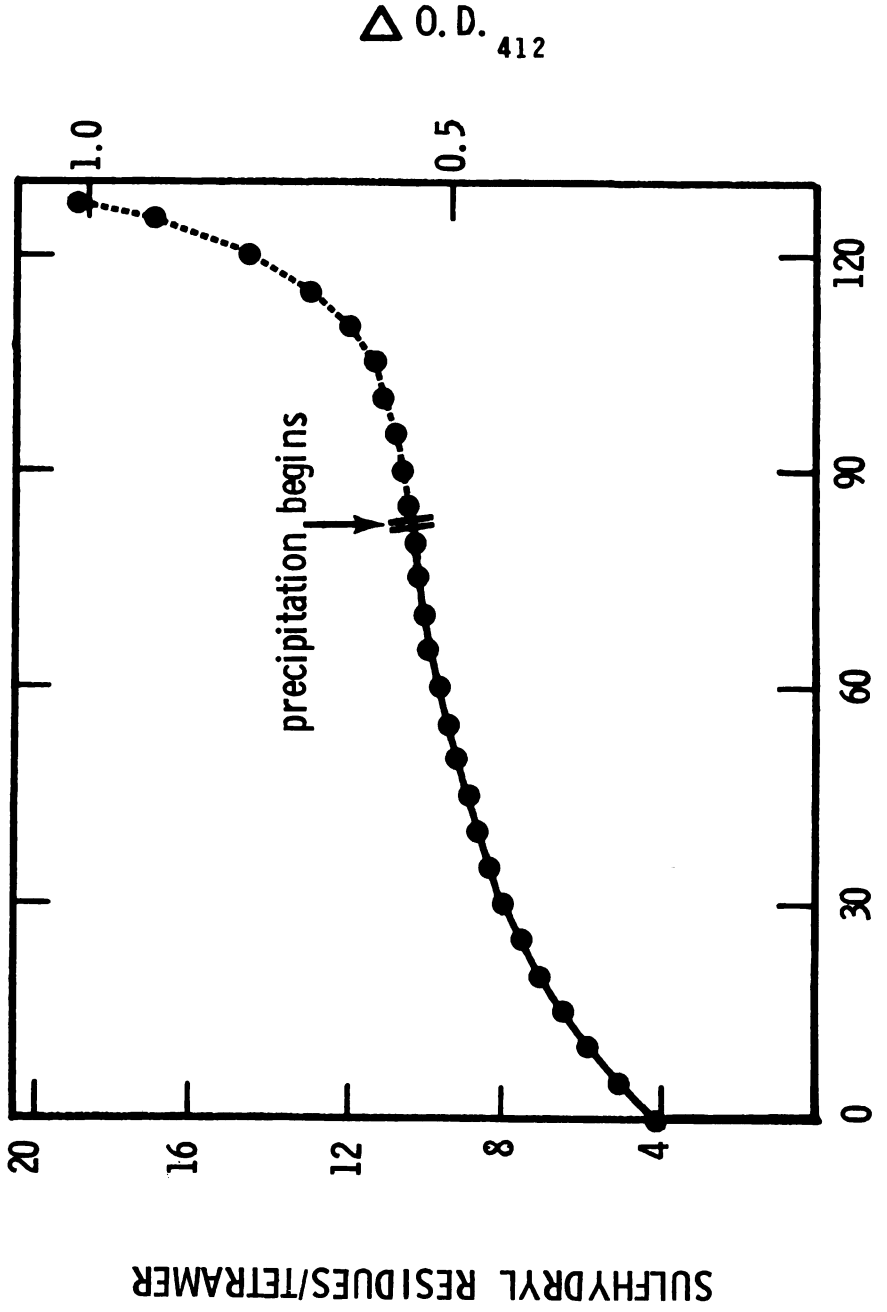
Δ O.D. 412
1.0
0.5
precipitation begins
0
30
60
90
120
TIME (MINUTES)
20
16
12
8
4
SULFHYDRYL RESIDUES/TETRAMER

Figure 20

rate (in approximately 30 min). Two additional sulfhydryl groups reacted very slowly, requiring 90 min from the beginning of the reaction to become fully titrated.

At approximately 90 min, GAPD began to denature and precipitate. Light scattering off this precipitate gave the final rapid rate observed between 90 and 130 min. (The precipitation was confirmed by observing the turbidity at 650 nm.) Whether the last six sulfhydryl groups were titrated during this rapid structural change was not determined, but it seems likely that these groups would have become available for titration during the unfolding which usually proceeds denaturation. It also seems probable that the two sulfhydryl groups titrated between 30 and 90 min were made available for titration by the enzyme slowly beginning to unfold.

DISCUSSION

EDTA EFFECTS AND THE POSSIBLE PRESENCE AND ROLE OF ZINC.

In previous work by Dagher and Deal (in preparation), pig liver GAPD had appeared to be partially resistant to dissociation and to show almost no reactivation. The original objective in this research was to determine why the characteristics of the dissociation of pig liver GAPD by ATP at 0° appeared to be so different from those of yeast and rabbit muscle GAPDs. The key discovery in solving this problem was finding that EDTA was required for producing an active dissociated enzyme and for reactivation. This discovery was especially important because in the previous work activity determination had been the only technique used to analyze for dissociated (by reactivating) and reactivated enzyme.

EDTA stabilized the activity of native pig liver GAPD and stabilized against activity loss in the dissociation system. When the enzyme was incubated at 0° with or without ATP, it retained more activity in the presence than in the absence of EDTA. And a comparison of enzyme dissociated by ATP at 0° with and without EDTA showed that in both cases the dissociation products were

similar in structure (based on their sedimentation coefficients), but the "dimer" in the absence of EDTA had much less activity.

The stabilization by EDTA suggests that GAPD is sensitive to metals and that the mechanism for this stabilization of enzymatic activity may be related to the ability of EDTA to chelate metals. One possible mechanism is the relief of heavy metal inhibition of catalytic activity. This mechanism is supported by Velick and Furfine's observation (1963) of the inhibition of GAPD by heavy metals and by Nagradova's studies (1965) on the increased activity of rabbit muscle GAPD in the presence of chelating agents. However, the mechanism appears unlikely because adding EDTA to native pig liver GAPD did not increase its activity. A second possible mechanism--the removal of metal ions whose presence on the enzyme disturb its structure enough to make it more susceptible to activity loss under inactivating conditions (low temperature or the presence of ATP at 0°) and to interfere with reactivation--seems quite plausible.

It is interesting to speculate about how these metal ions, that can be removed by EDTA, affect the activity of GAPD. From the data discussed in this thesis, it appears that the metal ions only affect reactivation and the activity of the dissociated enzyme; they do not affect dissociation or the activity of the native enzyme.

These selective effects suggest that there is a difference between the way that metal ions are bound to the dissociated and the native forms of the enzyme because the metal ions affect the behavior of the former, but not the latter. Polgar (1964) and Boross (1965) have discovered that metal ions can bind to GAPD through the sulfur atom of the cysteine residues. Ovadi, Nuridsany and Keleti (1973) have strong evidence that at least one cysteine residue per monomer which is normally buried in the tetrameric GAPD is exposed by dissociation in ATP at 0°. Consequently, it seems likely that the metal ions on GAPD could bind the sulfhydryl residues that are newly exposed by dissociation, causing the subunits to be bound together in an incorrect conformation and causing a decrease in the activity of the dissociated enzyme and in reactivation. (Heavy metal ions have numerous binding sites that are not always filled; for example, zinc can form tetrahedral and octahedral complexes.)

If indeed EDTA is chelating metal ions, the most likely source of these ions would be the $ZnCl_2$ precipitation step at the beginning of the purification procedure (Dagher and Deal, in preparation). The idea that pig liver GAPD could successfully carry bound zinc through the purification and crystallization is supported by Keleti's contention that zinc is firmly bound to a number of GAPDs (Keleti et al., 1962). And though Park

has convincingly argued that active GAPD does not require (or even have) bound zinc (Barkman, Sandstead and Park, 1970), the large amounts of data on zinc binding to GAPD gathered by Keleti (Keleti and Telegdi, 1959a,b; Keleti et al., 1962; Keleti, 1964, 1966) and the study of the mechanism of zinc binding to GAPD by Polgar (1964) strongly imply that zinc can bind to GAPD, even if the binding is nonphysiological.

Whether or not zinc is bound to the crystalline pig liver GAPD, our data support Park's theory that zinc is nonessential (Barkman, Sandstead and Park, 1970) rather than Keleti's idea that zinc is required for catalytic activity and the maintenance of steric structure (Keleti and Telegdi, 1959a,b; Keleti et al., 1962). For example, chelation by EDTA had no observable effect on the activity or structure of native GAPD. Dissociated GAPD had less activity in the absence than in the presence of EDTA. And GAPD without EDTA lost more activity at low temperature or in the presence of ATP at 0° than GAPD with EDTA.

Although zinc appears to be nonessential for normal functioning, the question of control of enzymatic activity by zinc should still be considered. There is approximately 12 μg of zinc per gram of tissue (wet weight) in the normal liver (Leucke, personal communication). Zinc could aid in decreasing the activity of

GAPD in the cell by binding to the dissociated enzyme and decreasing its activity. (GAPD in the absence of EDTA was more inactivated by low temperature or ATP at 0° than GAPD in the presence of EDTA.) Chelation of zinc and reversal of the action of the dissociating agents would reactivate the GAPD when full activity was required by the cell. (EDTA was required for reactivation.) But such a mechanism is negated by our data showing that chelation must occur before dissociation begins if reactivation is to be successful. (EDTA added just before warming did not effect reactivation.) However, since inactivation at low temperature is non-physiological, the significance of the mechanism and its negation cannot be assessed.

No experiments have been conducted to ascertain whether or not zinc actually is bound to crystalline pig liver GAPD purified by the method of Dagher and Deal (in preparation). Until such experiments are performed, it is impossible to be certain that the EDTA effects observed are related to the chelation of zinc.

DISSOCIATION INTO DIMERS. Based on the sedimentation coefficients of 4.9 to 5.1 S, the ATP-dissociated GAPD had a molecular weight of 74,900 to 77,200 daltons, assuming a globular protein. The expected dimeric molecular weight is 76,100 daltons, using the monomeric molecular weight calculated from the amino acid

composition. Therefore, the product of ATP dissociation under standard conditions is assumed to be a dimer.

Theoretically, the 4.9 to 5.1 S enzyme could be the result of a tetramer-monomer equilibrium, instead of a dimer. This possibility seems unlikely because although sedimentation coefficients intermediate between those of tetramer and dimer were seen, no sedimentation coefficients intermediate between those of dimer and monomer were seen (Figure 7). In a tetramer-monomer equilibrium, the entire spectrum of intermediate sedimentation coefficients would be expected. Constantinides and Deal (1969) also observed a tetramer-dimer equilibrium, but not a monomer-dimer equilibrium in sucrose density gradients with rabbit muscle GAPD dissociated by ATP at 0°. With sedimentation velocity experiments in the ultracentrifuge, they were able to resolve the peak, whose sedimentation coefficient was intermediate between those of tetramer and dimer, into two peaks whose sedimentation coefficients were identical to those of tetramer and dimer.

The peak with the sedimentation coefficient value of 6.8 S (Figure 7) is intermediate between the sedimentation coefficients expected for tetramer (7.8 S) and for a folded dimer (5 S). This observation of a single peak with a sedimentation coefficient intermediate between those of a tetramer and dimer requires

explanation. The simplest explanation is that a rapid (or at least a semi-rapid) equilibrium is established between the tetrameric and dimeric species. Furthermore, the sedimentation coefficient is a weight average quantity; for example, as concentration or other changes cause a redistribution of mass between dimer and tetramer, the weighted average sedimentation coefficient would reflect the change in distribution. In order for this rapid (or semi-rapid) equilibrium explanation to be the correct one, the rate of tetramer-dimer interconversion must be comparable to the rate of sedimentation in the gradient. The rate of enzymatic dissociation (10-12 hr) and the rate of tetramer-dimer equilibration do not have to be the same to observe a single peak; the mixture applied to the gradient is very near to equilibrium, so if partial resolution into dimeric and monomeric species were to occur, kinetic forces would cause material in both peaks to be converted into the other species. Under these conditions it is not surprising that we do not get resolution. In conclusion, we do not postulate that the peak with an intermediate sedimentation coefficient represents a system in complete rapid equilibrium; what we do postulate is that the occurrence of association-dissociation reactions results in sufficient mass sedimenting at a position intermediate between those of the tetramer and dimer to prevent resolution of the tetramer and dimer components.

Unlike ATP dissociation of yeast (Stancel and Deal, 1969) and rabbit muscle GAPDs (Constantinides and Deal, 1969), ATP dissociation of pig liver GAPD did not readily produce monomers. However, at the very low concentration of 0.025 mg/ml GAPD, dissociation produced 85 per cent monomers and 15 per cent dimers (Figure 7). Because the lower limit for our protein determination technique was 0.025 mg/ml and because the monomer (unlike the dimer) was inactive, we were unable to investigate whether even lower concentrations of GAPD would dissociate completely into monomers. The inactive monomer could not be reactivated using the standard conditions.

ENZYMATIC ACTIVITY OF DISSOCIATED GLYCERALDEHYDE 3-P DEHYDROGENASE. Our data suggest the existence of an active dimer of pig liver GAPD. The 4.9 to 5.1 S product from the standard inactivation-dissociation system was active at 0° after sucrose density gradient centrifugation. In sucrose gradients of dissociated GAPD, the sedimentation coefficient calculated from the activity determination was always that of a dimer, even when the sedimentation coefficient calculated from the protein determination was lower.

A comparison of dimer and tetramer specific activity indicates that the dimer may be 50 per cent as active as the tetramer. In the reassociation studies when reactivated GAPD was centrifuged in a gradient containing ATP, the dissociated species formed during centrifugation

was 50 per cent as active as the native enzyme centrifuged in a gradient without ATP. In the inactivation studies, the enzyme lost approximately 50 per cent of its specific activity under most conditions (exceptions: without EDTA, at low protein concentration, at pH 6.0 and at 25°), and analysis of the size of this partially inactivated enzyme in sucrose density gradients indicated that it was a dimer.

There is no evidence for an active monomer. The only monomer observed was found after inactivating GAPD at 0.025 mg/ml, and it had no activity.

The existence of an active dimer has been proposed by other workers. Constantinides saw indications of an active dimer in reassociation studies of ATP-dissociated rabbit muscle GAPD using sucrose gradients containing ATP and in inactivation studies at 7° (Constantinides and Deal, 1969). His data were similar to our data described above. Keleti's laboratory reported an active dimer of pig muscle GAPD produced by incubation with ATP at 0° in the presence of mercaptoethanol and isolated on a Sephadex G-100 column (Ovadi et al., 1971). The dimer had 50 per cent as much activity as the tetramer.

Active dimers have also been found in other types of dissociation systems. Elodi (1958) found active dimers after dissociation in KCN, and Kirschner and

Schuster (1970) found the same after dissociation in high ionic strength. However, the active enzyme sedimentation velocity studies of Hoagland and Teller (1969) using rabbit muscle GAPD dimers produced by dissociation at low temperature failed to show any active dimers. The fact that not all dissociated enzyme is active suggests that different dissociation techniques produce different conformations of dissociated enzyme with different activities.

COMPARISON OF INACTIVATION AND DISSOCIATION OF PIG LIVER GAPD WITH THOSE OF OTHER GAPDS. Comparing the inactivation and dissociation of pig liver GAPD with those of yeast GAPD (Stancel and Deal, 1968, 1969), few similarities are found. The pig liver enzyme dissociates to active dimers, the yeast enzyme to inactive monomers; both dissociated enzymes are fairly compact. Inactivation requires 12 hr for pig liver GAPD and only 50 per cent of the activity is lost; complete inactivation requires only 5 hr for yeast GAPD. Optimal protein concentration for both enzymes is 0.1 mg/ml; but concentrations as low as 0.03 mg/ml of yeast GAPD will give the same dissociated monomer, while 0.025 mg/ml of pig liver GAPD gives a mixture of monomers and dimers. The yeast monomers produced at both concentrations can be reactivated; the pig liver dimers produced at the higher concentration can be reactivated, but the monomers

produced at the lower concentration have not yet been reactivated. The pH at which the maximal rate and extent of inactivation and dissociation occur for yeast GAPD is pH 9.0; for pig liver GAPD it is a pH range of 7.5 to 9.0. The optimal ATP concentration for dissociation is 15 mM for the pig liver enzyme and 1-2 mM for the yeast enzyme. Both enzymes inactivate best at 0°; but pig liver GAPD still inactivates well at 12°, while yeast enzyme shows only 50 per cent inactivation. And both enzymes are normally inactivated in the presence of mercaptoethanol to permit reactivation.

Comparing the inactivation and dissociation of pig liver GAPD to those of rabbit muscle GAPD (Constantinides and Deal, 1969), several similarities are found. Both enzyme dissociate to active dimers at one protein concentration and to inactive monomers at a lower protein concentration. However, for pig liver the concentrations are 0.1 mg/ml and 0.025 mg/ml respectively, and for rabbit muscle they are 1.0 mg/ml and 0.1 mg/ml respectively. Also, rabbit muscle monomers can be reactivated, while pig liver monomers have not yet been reactivated. Both enzyme inactivate reversibly in the pH range 7.5 to 9.0; but the rabbit muscle enzyme still inactivates reversibly down to pH 6.5, while pig liver enzyme inactivates irreversibly at pH 6.0. Inactivation of rabbit muscle GAPD takes two hours and results in a

90 per cent loss of activity; inactivation of pig liver GAPD takes 12 hr and results in a 50 per cent loss of activity. Both enzymes inactivate best at 0°; but pig liver enzyme still inactivates at 12°, while rabbit muscle enzyme does not. Optimal ATP concentrations for dissociation are 2 mM ATP for rabbit muscle GAPD and 15 mM ATP for pig liver GAPD. Both enzymes are normally inactivated in the presence of mercaptoethanol to permit reactivation.

The conditions required for inactivation and dissociation of pig muscle GAPD have not been carefully characterized (Ovadi et al., 1971), making it difficult to compare its inactivation and dissociation with those of pig liver GAPD. However, the following points can be noted. In both cases the process can occur at 0° and pH 8.5. Pig muscle GAPD at 1.4 mg/ml will dissociate to dimers at ATP concentrations as low as 0.4 mM ATP; pig liver GAPD at 0.1 mg/ml requires 10 mM ATP to dissociate to dimers. Both enzymes give active dimers only if mercaptoethanol is present. Under maximal inactivation conditions, complete inactivation is obtained in 5 hr with pig muscle GAPD and in 12 hr with pig liver GAPD.

In summary, the inactivation and dissociation of pig liver GAPD are more similar to those of pig muscle and rabbit muscle GAPDs than to those of yeast GAPD.

This trend is not surprising since the former three are mammalian sources while the latter is microbial.

COMPARISON OF REACTIVATION AND REASSOCIATION OF PIG LIVER GAPD WITH THOSE OF OTHER GAPDS. There are almost no similarities in the reactivation and reassociation of pig liver GAPD and those of yeast GAPD (Stancel and Deal, 1969). Yeast GAPD reactivates at 17°, pig liver at 25°. Reactivation of yeast GAPD takes one hour; reactivation of pig liver GAPD takes five min. Yeast enzyme requires 1-2 mM ATP and 10 per cent sucrose for reassociation; pig liver enzyme requires 5 mM NAD and 0.1 M EDTA, and ATP and sucrose hinder the reassociation. But both enzymes require mercaptoethanol to reassociate. And both enzymes show decreasing reactivation with increasing periods of inactivation.

Many similarities are observed in the reactivation and reassociation behavior of pig liver GAPD and that of rabbit muscle GAPD (Constantinides and Deal, 1969). Both enzymes reactivate at room temperature, and reactivation takes approximately 5 min. Both enzymes require mercaptoethanol for reassociation, and reassociation is hindered by ATP. With both enzymes, the centrifugation of reactivated enzyme in a sucrose gradient containing ATP causes the appearance of a dissociated enzyme. The only difference is the requirement of 0.1 M EDTA and 5 mM NAD by pig liver GAPD for reactivation; rabbit muscle GAPD has no such requirements.

In summary, the reactivation and reassociation characteristics of pig liver GAPD are very similar to those of rabbit muscle GAPD and very different from those of yeast GAPD. The similarities and differences are like those for inactivation and dissociation.

COMPARISON OF AMINO ACID COMPOSITION WITH THAT OF OTHER GAPDS. A comparison of the amino acid composition of pig liver GAPD with those of pig muscle (Harris and Perham, 1968), rabbit muscle (Harris and Perham, 1963; Velick and Furfine, 1963; Allison and Kaplan, 1964), yeast (Jones and Harris, 1972), lobster muscle (Davidson et al., 1967), human muscle (Allison and Kaplan, 1964; Wolny, 1968), chicken muscle, Escherichia coli and halibut muscle GAPDs (Allison and Kaplan, 1964) revealed that pig liver GAPD has approximately the same ratio of acidic, basic and hydrophobic amino acids as all the other GAPDs. However, pig liver GAPD may have 9 to 17 more residues per monomer than the other enzymes. Comparing the amounts of individual amino acids found in all the different GAPDs, pig liver GAPD has 30 per cent more methionine and 60 per cent more proline than the other GAPDs; it also has slightly more glycine (as does human muscle GAPD). The amounts of cysteine, aspartic acid and serine in pig liver GAPD are like the amounts found in other mammalian GAPDs. The number of threonines, valines, phenylalanines and histidines in

pig liver enzyme are similar to the numbers in all the vertebrate enzymes and in lobster muscle GAPD. And all the enzymes including pig liver GAPD have approximately the same amount of glutamic acid, alanine, isoleucine, leucine, tyrosine, lysine and arginine. Pig liver GAPD also has a small number of tryptophans as is common for all GAPDs (except E. coli), but, like rabbit muscle GAPD, it has four or five residues rather than the typical three.

This strong conservation of the amino acid composition of GAPD, even among phylogenetically distant species, suggests a selection against variations produced by mutations. Apparently, this enzyme, as a member of the basic pathway for converting carbohydrates to energy and producing storable complex sugars, is so important that the cell will not tolerate alterations of its specificity and structure.

COMPARISON OF SULFHYDRYL REACTIVITY OF PIG LIVER GAPD WITH THAT OF OTHER GAPDS. In pig liver GAPD, one cysteine per monomer reacts rapidly, a second reacts at a moderate rate and the two remaining cysteines are buried (observed by titration with DTNB). This pattern of sulfhydryl reactivity is the same as that observed with pig muscle GAPD (titrated by PCMB) (Boross, Cseke and Vas, 1969; Vas and Boross, 1970) and with lobster muscle GAPD (titrated by organic mercurials) (Wassarman, Watson and Major, 1969).

The extremely reactive cysteine residue is probably Cys-149, a major functional group in the active site; other workers have established a connection between the titration of one very reactive cysteine and the inactivation of GAPD, and have identified the cysteine involved (Boross and Cseke, 1967; Szabolcsi, Biszuku and Sajgo, 1960; Bernhard and MacQuerrie, 1971). The moderately reactive cysteine is most likely Cys-153 as proposed by Vas and Boross (1970) from their work on PCMB titration of pig muscle GAPD. The buried cysteines are probably Cys-281, the sulfhydryl group exposed after ATP dissociation of pig muscle GAPD into dimers (Ovadi, Nuridsany and Keleti, 1973), and Cys-244, which is exposed only after the enzyme is unfolded.

In rabbit muscle (Smith and Schachman, 1971) and pig muscle GAPDs (Szabolcsi, Biszuku and Sajgo, 1960; Friedrich and Szabolcsi, 1967), the titration of two sulfhydryls per monomer with PCMB caused the enzyme to unfold and expose the buried sulfhydryls. In pig liver GAPD, the titration of two sulfhydryls per monomer with DTNB caused the enzyme to precipitate. Since precipitation can result when unfolded protein aggregates, it seems likely that titration of two cysteines did cause pig liver GAPD to unfold. But because the precipitate caused so much light scattering, it is impossible to tell whether the buried sulfhydryls

were exposed and titrated. However, the titration of one cysteine on two of the subunits (reaction between 30 and 90 min) before the precipitate became too heavy suggests that at least some of the buried cysteines were being exposed and titrated.

The nearly identical sulfhydryl reactivity of native enzyme seen with all GAPDs studied is even more strongly conserved than the overall amino acid composition of GAPD. This strong conservation indicates that the position of some or all of the sulfhydryl residues is extremely important to the maintenance of the structure and activity of the enzyme.

SUMMARY AND CONCLUSIONS

The main accomplishments in this research were (1) the complete dissociation of liver GAPD by ATP and the characterization and optimization of this process, (2) the discovery of the right conditions for reactivation and reassociation, and (3) the discovery of the sensitivity to metals which is so critical for reactivation and for activity of the dissociated enzyme.

Pig liver GAPD was inactivated and dissociated by ATP at 0°. The optimal conditions were 0.1 mg/ml GAPD, pH 7.5 to 9.0, 15 mM ATP and 0°. Under these conditions the process required 10 to 12 hr. At low pH values or low protein concentrations, the native enzyme dissociated at 0° in the absence of ATP. The dissociated product was a dimer with a sedimentation coefficient of 4.9 to 5.2 S; if it was produced in the presence of 0.1 M EDTA and 40 to 80 mM mercaptoethanol, the dimer was active.

The inactivated and dissociated enzyme was reactivated by simply warming to 25°. Reactivation required 3.5 to 5 min. The optimal conditions for

reactivation were 0.1 M EDTA, 80 mM mercaptoethanol, 25°, 5 mM NAD and no sucrose. In the absence of ATP, the reactivated GAPD reassociated to a tetramer with a sedimentation coefficient of 7.8 S (same as that of native GAPD).

The amino acid composition of pig liver GAPD was found to be similar to that of other GAPDs, especially the mammalian enzymes. The sulfhydryl reactivity of native pig liver GAPD is also very similar to the reactivity of other GAPDs.

The requirement of EDTA for the production of active dimers and for reactivation of the dissociated enzyme suggests that EDTA chelates a metal ion(s) on the enzyme. The metal ion could easily be zinc picked up during purification. From our data it was determined that zinc, if present, is nonessential, but it was speculated that zinc could play a possible role in an enzymatic control mechanism.

The physiological significance of reversible dissociation and inactivation at 0° and low protein concentrations in the presence of high levels of chelating agent is questionable. But the importance of this research lies not in its questionable physiological significance, but in its commentary on the forces required to maintain the native structure of GAPD. These forces have been discussed by Stancel (1970) in

his study on reversible inactivation and dissociation of yeast GAPD by ATP at 0°. The binding of ATP disturbs the steric structure and exposes a part of the hydrophobic core of the enzyme. These hydrophobic bonds are destabilized by low temperature, and the enzyme dissociates. Warming the enzyme to 25° permits the hydrophobic bonds to reform. Mercaptoethanol is required to keep disulfide bonds from forming (Ovadi, Nuridsany and Keleti, 1973), and EDTA keeps zinc from binding to the cysteine residues exposed by dissociation (Polgar, 1964); otherwise, reactivation and reassociation could not occur.

Further experiments suggested by this research are (1) an analysis of whether or not zinc is present on crystalline pig liver GAPD, (2) a discovery of the source of the zinc if it is present, (3) an examination of possible GAPD dissociation at 25° and 37° (low protein concentration), (4) a search for the dissociation conditions required at high protein concentrations, (5) a study to see whether or not the catalytic properties of the dissociated product, active dimer, differ from those of native tetramer, (6) a search for the conditions required for complete dissociation into monomers, (7) a search for the conditions required to reactivate monomers, (8) a study of the dissociation of NAD-free enzyme to see if it requires less ATP than enzyme with bound NAD, (9) an analysis of the effect of various

EDTA concentrations on reactivation and production of active dimers, and (10) a study of the possible reversible dissociation of pig liver GAPD by other dissociating agents such as KCl, $(NH_4)_2SO_4$ and urea.

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